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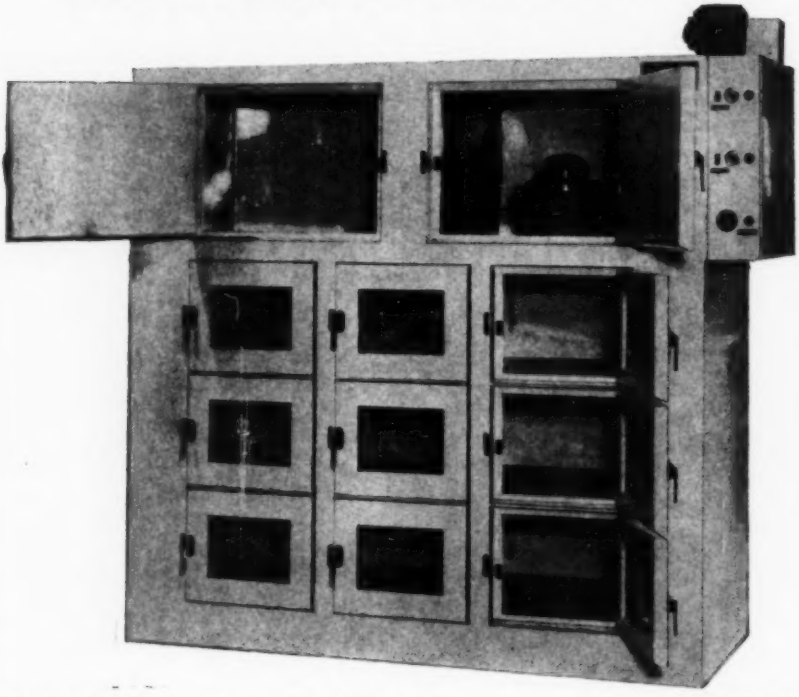

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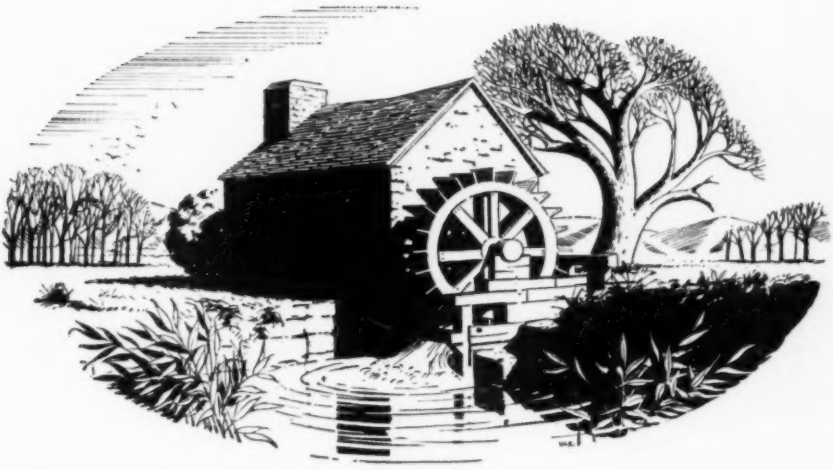

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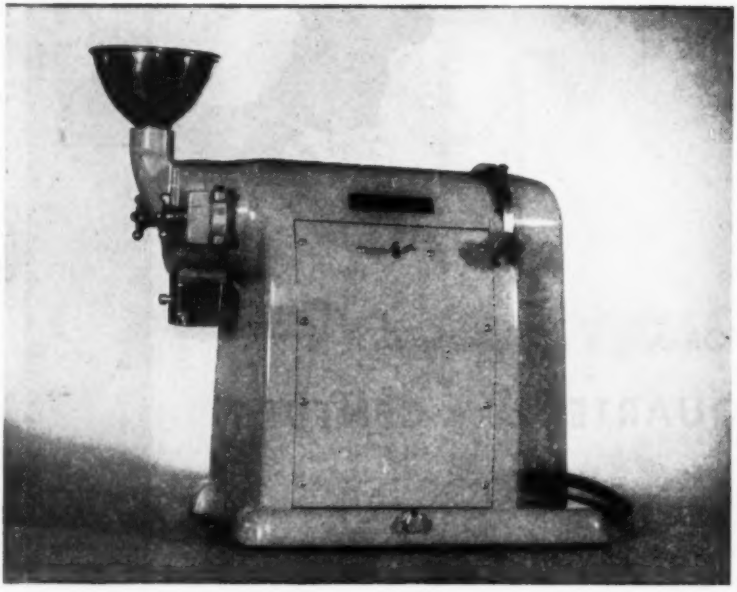

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CEREAL CHEMISTRY

VOL. XXIX MAY, 1952 No. 3

EFFECT OF MOISTURE ON THE MICROFLORA AND FORMATION OF FREE FATTY ACIDS IN RICE BRAN[1, 2, 3]

JOSEPHINE R. LOEB AND RUTH Y. MAYNE

ABSTRACT

Factors influencing the deterioration of rice bran during storage were investigated by comparing the effect of moisture content on the growth of bacteria and of filamentous yeasts and molds with its effect on the formation of free fatty acids in the oil. High moisture content had a similar effect upon microorganisms and formation of free fatty acids and both often increased at the same time in the same sample. The growth of *Aspergillus chevalieri*, one of the molds present in untreated bran, produced free fatty acids in rice bran sterilized by autoclaving. However the rate of production of free fatty acids was slower than in untreated bran. In relatively dry, unautoclaved bran (11.5% moisture), free fatty acids were formed without a detectable increase in microorganisms. There was even a slow formation of fatty acids in bran with moisture content as low as 4.9%.

Hydrolysis of the glycerides of the oil in rice bran to form free fatty acids is one of the principal causes of deterioration of the bran during storage (14). This process is generally considered to be the result of enzyme activity. Browne (5) reported that lipase is present in rice bran and that heating the bran to 90°C. partially inhibited lipolytic action. West and Cruz (25) reported that the formation of free fatty acids resulted from the action of moisture catalyzed by enzymes and that the most convenient method to preserve rice bran was to heat it at about 105°C. for three hours and store in moisture-proof packages. They emphasized the importance of moisture and insects in the deterioration of bran but, on the basis of a limited investigation, dismissed bacteria as a cause of the spoilage.

Loeb *et al.* (14) found that dry heat up to 110°C. for two hours effectively inhibited the formation of free fatty acids in the oil, by removing the moisture required for hydrolysis, since the activity re-

[1] Manuscript received December 10, 1951.
[2] Report of a study carried on under the Research and Marketing Act of 1946.
[3] Contribution of Southern Regional Research Laboratory, New Orleans, Louisiana. One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

curred when moisture was added. In an unpublished experiment by Loeb, it was indicated that in rice bran sterilized by autoclaving, no free fatty acids were formed in storage until microbial contamination occurred.

Roberts *et al.* (19) showed that the formation of free fatty acids in stored bran was inhibited by preliminary steam treatment at 212°F. (100°C.) for as little as one minute in a continuous blancher, but they did not report the moisture content nor the microflora present during storage. However, they commented on the high count of microorganisms in unheated bran and the possibility that these might furnish some or all of the lipase activity.

While the references just given are those pertaining to rice bran, many workers have investigated the growth of microorganisms and deterioration in other materials. For example, the concomitant growth of microorganisms and increase in fat acidity was noted by Milner *et al.* (17) and Carter and Young (6) in wheat, Bottomley *et al.* (3, 4) in corn, Milner and Geddes (16) in soybeans and Christensen *et al.* (8) in cottonseed; a review of the work on corn was written by Semeniuk and Gilman (20).

Since it is known that various molds and bacteria can produce lipase (9, 15, 23), and that molds can grow at the temperatures and humidity usually prevailing where rice bran is stored in bulk (2, 10, 22), it was considered important to determine whether the growth of microorganisms has a major effect upon the rate of lipolysis during storage. This information might determine, for practical purposes, whether aseptic storage or storage in some condition inhibiting microbial growth, such as an extremely low moisture content, is necessary after an inactivating treatment.

The purpose of the work reported here was to determine in what way the moisture content of the bran would affect the proliferation of microorganisms and the formation of free fatty acids during storage, and whether microbial activity could contribute to the formation of free fatty acids in the bran oil.

Materials and Methods

Regularly milled bran from Southwestern long-grained rice, either Rexora or Bluebonnet variety, was used. For each experiment it was handled promptly before any appreciable deterioration could occur.

General Plan. Four experiments were carried out to observe the microflora and the formation of free fatty acids during storage of fresh bran having four variations in preparation. In the first experiment, bran of natural moisture content was stored at different relative humidities, and observations were made during equilibration of the moisture

content of each sample to the surrounding humidity. In the second experiment, before storage the moisture contents of the bran samples were adjusted to approximate hygroscopic equilibria with the same relative humidities as in the first experiment. It was recognized that this artificially adjusted moisture might exist in a different physical state from the natural content. However, it was believed that this possibility would not invalidate the results. The third experiment was like the second except that the bran was autoclaved before the moisture contents of portions were adjusted. In the fourth experiment, autoclaved samples were adjusted to three different moisture contents and inoculated with mold spores before being stored at the relative humidities which were in hygroscopic equilibria with the samples. As controls, three autoclaved uninoculated samples and three unautoclaved samples were stored at corresponding moisture contents and relative humidities.

In all four experiments, the samples were stored in sterile crystallizing dishes which were placed in desiccators maintained at the desired relative humidities by the presence of saturated salt solutions (11). The desiccators were stored in a room maintained at a constant temperature of 70°F. (21.1°C.)—the only available constant temperature room. Although this temperature is low in the range for microbial growth and for usual storage practice, it was suitable for these experiments since production of free fatty acids occurs rapidly at even lower temperatures (14). At least one bran sample in each experiment was stored in a desiccator without humidifying solutions to serve as a control. All procedures were conducted aseptically.

At various intervals in all experiments the bran in each dish was thoroughly mixed and a sample was removed for analyses. Values for moisture, reported here on a wet weight basis (Bc 2-49), and for free fatty acids (Aa 6-38) were obtained by the official methods of the American Oil Chemists' Society (1) for oilseeds except that the bran was not ground and the free fatty acids were determined on approximately 3 g. samples of extracted oil.

Microbial examination consisted of making plate counts of viable molds and filamentous yeasts and of bacteria, and identification of the predominant types of molds. Identification of bacteria, which is more involved, was omitted. Serial dilutions of a 5 g. sample of bran suspended in water and mixed for one minute at top speed in a Waring Blendor[4] were plated. In the first experiment wort agar was used as a culture medium in determining the numbers of viable molds and filamentous yeasts present. However the interference of bacterial growth necessitated an exploratory experiment to find a medium more selective

[4] The use of trade names in this article is for identification and implies no endorsement of the manufacturer or the product.

for the molds and filamentous yeasts occurring in rice bran. Of five agar media, Waksman's (21), malt salt (7), potato dextrose with tartaric acid, wort, and acid dextrose Czapek's (13), the first three were best in preventing growth or spread of bacterial colonies. Waksman's and malt salt gave the highest counts of molds and filamentous yeasts. Waksman's medium was selected because in this experiment with rice bran it was observed to support the growth of more varieties of molds. Tryptone glucose extract agar (Difco[4]) was used in determining the number of bacteria. Counts of microorganisms were made after incubation at 26°C. for three days. Molds were transferred and identified by their characteristics in pure culture on potato dextrose and Czapek's sucrose agar (24, p. 32).

Experiment Number 1. Bran of 12.3% moisture from Bluebonnet variety rice was stored at seven relative humidities: 48.8, 59.0, 65.2, 75.5, 80.3, 93.1, and 100%. One control was stored in a desiccator without humidifying solution.

Experiment Number 2. The same seven relative humidities were used with bran from Bluebonnet variety rice, but the moisture contents of the bran samples were adjusted before storage to the amounts which, with one exception, had been ascertained in the first experiment to be in approximate equilibrium with the humidities: namely, moistures of 8.6, 9.8, 10.7, 13.7, 13.7, 21.3, and 29.8%, for the seven humidities, respectively. The exception was the use of 13.7% moisture at 80.3% relative humidity, because the value observed in the preceding experiment appeared too low to be in relationship with the other values. Since the original moisture content of the bran was higher than three of the required values, all the bran, except for a portion kept as a control, was dried by heating in shallow layers for three hours at 85°C. Preliminary investigations had shown that this amount of heating neither prevented the formation of free fatty acids nor destroyed a significant number of microorganisms. After cooling and mixing, the bran was divided into eight equal portions, seven of which were rehydrated with sterile water as required and then each was stored at its appropriate relative humidity. The eighth portion was stored as a heated control in a desiccator without humidifying solution as was the unheated control.

Experiment Number 3. Bran from Rexora variety rice was autoclaved at 15 lbs. of pressure (121°C.) for two hours in shallow trays, dried for one hour at 60°C. and left overnight at room temperature to dry further. The bran was then thoroughly mixed and divided into five portions, each of which was placed in a crystallizing dish. Four samples were rehydrated with sterile water to the particular moisture contents found in Experiment Number 2 to be in equilibrium with the

four relative humidities: namely, moistures of 10.7, 13.7, 14.6, and 26.4% for the four relative humidities, 65.2, 75.5, 80.3, and 93.1%. The fifth autoclaved portion and also an unautoclaved portion with 11% and 11.8% moisture contents, respectively, were stored in dry desiccators without humidifying solutions as controls.

Experiment Number 4. In this experiment a strain of *Aspergillus chevalieri* (Mangin) Thom and Church, isolated from the bran used in Experiment Number 2 from Bluebonnet variety rice, was used because, although its optimum temperature is around 30°C., it grew well at 21.1°C. and 80.3% relative humidity, and was the predominant organism in the sample of bran which developed a high free fatty acids content.

Bran from Rexora rice, packaged in paper bags, was sterilized by autoclaving for three hours at 15 lbs. of pressure (121°C.), was then dried for three hours at 85°C., and cooled. Six portions were dispensed into six sterile crystallizing dishes. To three of these, calculated amounts of sterile distilled water were added to give moisture contents of 11.5, 14.5 and 33.5%, respectively, and a spore suspension was added to give a count of 53,000 spores per gram. The moisture contents of the bran in the remaining three dishes were similarly adjusted, but no spores were added. A third set of three sterile dishes were filled with bran which had not been autoclaved, and water was added to obtain the two higher moisture contents (14.5% and 33.5%), the lower content (11.5%) being naturally present in the bran. All nine samples were placed in desiccators, each at the relative humidity in equilibrium with the moisture content of the bran; that is, bran samples having 11.5% moisture content were placed in desiccators without humidifying solutions; samples having the other two moisture contents were placed in desiccators at humidities of 80.3% and 100%, respectively.

Results

Effect of Different Relative Humidities on Bran with Unadjusted Moisture Contents. In the experiment in which bran was stored without additional moisture at seven relative humidities the moisture contents of the samples and their respective contents of free fatty acids of the oils after 14 days of storage were:

Moisture Content of Bran	*Free Fatty Acids*
%	%
10.86	54.2
11.42	57.7
11.88	57.2
12.27	61.4
12.33	61.7
12.88	63.2
15.1	70.6

The control, which was kept in a desiccator without humidifying solution, had 11.9% moisture and 59.8% free fatty acids content. In general, the higher the relative humidity of storage, and consequently the higher the moisture content of the bran, the greater was the rate of formation of free fatty acids.

In 14 days, microorganisms had increased in only the sample having the highest moisture content. The change was from 160,000 to 1,300,000 molds and filamentous yeasts per gram; the predominating organism was a yeast, *Endomycopsis*.

Effect of Different Relative Humidities on Bran with Adjusted Moisture Contents. In the experiment in which the moisture contents of

TABLE I

VIABLE MOLDS AND FILAMENTOUS YEASTS IN THOUSANDS PER GRAM IN RICE BRAN[1] AFTER STORAGE AT DIFFERENT MOISTURE CONTENTS

Initial Moisture Contents[2] and Relative Humidities of Storage		Length of Storage, Days								
Moisture	Relative Humidity	0	3	7	10	14	17	24	35	45
%	%									
10.2[3]	[4]	24	25	18	17	31	27	23	—	—
4.9	[5]	7	5	4	7	6	7	6	—	—
8.6	48.8	—	7	7	7	6	7	19	—	—
9.8	59.0	—	8	10	9	7	8	12	—	—
10.7	65.2	—	7	3	8	8	7	15	—	—
13.7	75.5	—	8	3	149	320	174	620	330	390
13.7	80.3	—	8	27	530	430	960	1,130	4,800	3,950
21.3	93.1	—	7	5	28	11	12	480	3	19
29.8	100	—	5	3	2	1	1	<1	<1	<1

[1] Bran was dried for 3 hours at 85°C. in 1 to 1.5 in. layers in trays before storage.
[2] Reported on a wet weight basis.
[3] Unadjusted moisture of unheated control.
[4] Relative humidity existing without humidifying solution, about 65% according to data of Karon and Adams (12).
[5] Relative humidity existing without humidifying solution about 10% (12).

bran samples were adjusted, there was little change in moisture at relative humidities of 75% and below during storage for 24 days. This indicated that the moisture contents of the bran samples had been adjusted to coincide with the hygroscopic equilibria corresponding to the relatives humidities used. However, in samples stored at the relative humidities above 75%, the moisture contents changed. At 80.3% relative humidity, the moisture content after 24 days was 14.6% (originally 13.7%); at 93.1% relative humidity it was 26.4% (originally 21.3%) and at 100%, it was 33.5% (originally 29.8%).

Molds and filamentous yeasts increased greatly in numbers at 75.5% and 80.3% relative humidities, whereas the bacteria increased

most at 100% relative humidity (Tables I and II). The moderate heating used to dry the bran only slightly reduced the numbers of microorganisms.

The types of molds predominating changed during storage of the bran. *Mucor* sp. and the *Aspergillus flavus-oryzae* gr. were the most numerous originally. On the tenth day *A. chevalieri* was found in large numbers and later became predominant in the samples stored at 75.5% and 80.3% relative humidities; another *Mucor* sp. and a *Rhizopus* sp. were predominant in the samples stored at 93.1% and 100% relative humidities.

Changes in the bacterial flora, in so far as determined, were not as pronounced as those in the molds. The bacterial colonies remained

TABLE II

VIABLE BACTERIA IN MILLIONS PER GRAM IN RICE BRAN[1] AFTER STORAGE AT DIFFERENT MOISTURE CONTENTS

Initial Moisture Contents[2] and Relative Humidities of Storage		Length of Storage, Days								
Moisture	Relative Humidity	0	3	7	10	14	17	24	35	45
%	%									
10.2[3]	[4]	4	4	3	1	2	3	2	—	—
4.9	[5]	3	1	3	2	3	2	1	—	—
8.6	48.8	—	2	2	1	7	2	1	—	—
9.8	59.0	—	2	4	3	2	7	3	—	—
10.7	65.2	—	1	34	3	2	7	48	—	—
13.7	75.5	—	640	62	42	242	13	24	151	42
13.7	80.3	—	71	116	184	630	136	159	34	75
21.3	93.1	—	450	315	100	350	55	124	118	232
29.8	100.0	—	1,860	775	1,165	2,680	1,970	1,030	177	12

[1] Bran was dried for 3 hours at 85°C. in 1 to 1.5 in. layers in trays before storage.
[2] Reported on a wet weight basis.
[3] Unadjusted moisture of unheated control.
[4] Relative humidity existing without humidifying solution, about 65% (12).
[5] Relative humidity existing without humidifying solution, about 10% (12).

the same morphological types in all except the two samples stored at the highest humidities. In these two, after 10 days, changes occurred but the predominant organisms were not identified.

The higher the relative humidity of storage, the greater was the initial rate of formation of free fatty acids (Table III). The rates of increase of free fatty acids in an adjusted sample having approximately the same moisture content as the control and in the control were the same, suggesting that the method of addition of water did not influence the hydrolysis.

Effect of Moisture on Autoclaved Bran. The autoclaved control and the autoclaved samples stored at 10.7% and 13.7% moisture contents

TABLE III

FREE FATTY ACIDS IN THE OIL[1] OF RICE BRAN[2] AFTER STORAGE AT DIFFERENT MOISTURE CONTENTS

Initial Moisture Contents[3] and Relative Humidities of Storage		Length of Storage, Days								
Moisture	Relative Humidity	0	1	3	7	10	14	24	35	45
%	%									
10.2[4]	[5]	3.3	5.7	8.5	13.7	17.6	21.8	31.5	—	—
4.9	[6]	4.9	5.0	5.1	5.5	5.7	6.1	6.9	—	—
8.6	48.8	—	6.6	8.1	11.0	13.3	15.8	22.2	—	—
9.8	59.0	—	6.8	9.1	13.8	17.9	21.2	29.9	—	—
10.7	65.2	—	6.7	10.3	16.7	21.3	27.1	38.0	—	—
13.7	75.5	—	8.8	15.8	26.5	33.0	41.9	60.2	71.5	78.1
13.7	80.3	—	8.7	15.7	26.1	38.1	47.7	71.4	82.6	85.4
21.3	93.1	—	9.6	18.6	31.7	39.0	48.3	57.2	58.4	60.3
29.8	100	—	12.4	19.2	28.7	33.8	36.7	47.1	52.9	57.8

[1] Oil content of the untreated bran was 16.14%; moisture content 10.2%.
[2] Bran was dried for 3 hours at 85°C. in 1 to 1.5 in. layers in trays before storage.
[3] Reported on a wet weight basis.
[4] Unadjusted moisture of unheated control.
[5] Relative humidity existing without humidifying solution, about 65% (12).
[6] Relative humidity existing without humidifying solution, about 10% (12).

TABLE IV

CHANGES IN MICROBIAL CONTENTS AND FREE FATTY ACIDS DURING THE STORAGE OF RICE BRAN PREVIOUSLY AUTOCLAVED FOR TWO HOURS

Length of Storage	Initial Moisture Contents[1] and Relative Humidities of Storage								
	Moisture, % 11.8[2]			13.7	14.6		26.4		
	Relative Humidity, %[3]			75.5[4]	80.3[5]		93.1		
	F.F.A.[6]	Molds and Filamentous Yeasts	Bacteria	F.F.A.	F.F.A.	Molds and Filamentous Yeasts	F.F.A.	Molds and Filamentous Yeasts	Bacteria
days	%	× 10^3/g.	× 10^3/g.	%	%	× 10^3/g.	%	× 10^3/g.	× 10^3/g.
0	5.6	144	23	2.3	2.3	—	2.3	—	—
6	13.2	285	29	2.3	2.2	0^7	12.6	0.15	0^7
13	20.7	116	9	2.6	2.8	0^7	18.5	1	0.02
20	26.7	101	17	2.6	3.1	0.1	25.3	10	0.5
27	31.8	140	8	2.6	18.1	155	79.4	100	—
34	—	—	—	2.7	38.2	1,100	—	—	—
41	—	—	—	2.6	—	—	—	—	—

[1] Reported on a wet weight basis.
[2] Unadjusted moisture of unheated control.
[3] Relative humidity existing without humidifying solution, probably about 68%.
[4] Bran remained sterile although desiccator was opened for sampling.
[5] No bacterial contamination was found.
[6] F.F.A. denotes free fatty acids.
[7] Lowest dilution plated was 1:20.

remained sterile and no free fatty acids developed during the period of storage. The bran stored with 14.6% moisture (at 80.3% relative humidity) remained sterile and no free fatty acids formed for 13 days (Table IV), but after the 13th day contamination occurred and a mold of the *A. glaucus* group grew and free fatty acids increased. Bacterial contamination was not found. In the bran stored with 26.4% mois-

TABLE V

CHANGES OCCURRING IN MOLD COUNTS AND CONTENTS OF FREE FATTY ACIDS IN RICE BRAN STORED WITH DIFFERENT MOISTURE CONTENTS AND TREATMENTS

Length of Storage, Days	Initial Moisture Contents[1] and Relative Humidities of Storage								
	Moisture, % 11.5			14.5			33.5		
	Relative Humidity, %[2]			80.3			100		
	Unautoclaved Bran	Autoclaved Bran	Autoclaved Bran + *A. chevalieri*	Unautoclaved Bran	Autoclaved Bran	Autoclaved Bran + *A. chevalieri*	Unautoclaved Bran	Autoclaved Bran	Autoclaved Bran + *A. chevalieri*
	Molds and Filamentous Yeasts (10^3/g. of Bran)								
0	11	0[3]	—	—	—	—	—	—	—
1	9	0[3]	1	7	0[3]	14	33	0[3]	4
2	33	0[3]	62	40	—	8	340	—	2
3	14	0[3]	8	35	0[3]	1,285	840	0[3]	5,200
6	7	0[3]	8	37	0[3]	4,250	2,100	0[3]	5,900
13	7	0[3]	295	1,000	0[3]	560	10	0[3]	3,900
20	6	0[3]	4,600	8,700	—	3,800	—	—	—
	Free Fatty Acids in Oil of the Bran, %								
0	5.2	2.3	—	—	—	—	—	—	—
1	6.5	2.6	2.9	7.6	2.9	2.9	9.4	2.6	3.2
2	7.7	—	2.7	9.2	—	2.5	12.6	—	2.9
3	8.8	2.4	2.9	11.8	2.8	3.4	15.0	2.8	3.3
6	12.5	2.7	3.4	17.6	2.4	15.2	20.4	2.7	7.0
13	18.7	2.7	4.9	30.9	2.8	40.3	27.4	2.8	32.6
20	24.5	2.4	25.8	55.5	—	59.0	—	—	—

[1] Reported on a wet weight basis.
[2] Relative humidity existing without humidifying solution, probably about 68%.
[3] Lowest dilution plated was 1:20.

ture content, molds (*Rhizopus* sp.) were detected on the sixth day of storage, the day the first analyses were made, accompanied by an increase in free fatty acids. During storage the molds continued to multiply and on the thirteenth day bacteria were also observed (Table IV).

Effect of A. chevalieri on Free Fatty Acid Formation in Autoclaved Bran Stored at Different Moisture Contents. In the fourth experiment

on autoclaved-uninoculated, autoclaved-inoculated, and unautoclaved bran, the moisture content of each lot remained essentially at its initial value, original or adjusted.

The autoclaved, uninoculated lots remained sterile throughout the period of storage (13 days for those stored with 14.5% and 33.5% moisture contents and 20 days for the one with 11.5% moisture). Table V shows that during these periods the content of free fatty acids did not increase, as was the case in the experiment with autoclaved bran only.

In contrast, the other two sets of bran lots, that is, the unautoclaved and the autoclaved-inoculated bran, showed marked changes under each of the three conditions of storage. In unautoclaved bran with 11.5% moisture, the bacteria, originally 15 million, remained the same in numbers and types of colonies during the storage period. The total number of molds also remained the same. Those appearing originally had been *Mucor* sp., *Penicillium* sp., and *Aspergillus flavus-oryzae*, but on the sixth day *A. candidus* predominated in the mixture. The free fatty acids of the bran oil increased 3% in the 24 hours between receipt at the laboratory and the time of storage at 21.1°C.; they continued to form at a slower rate under the conditions of the experiment. On the other hand, in the autoclaved-inoculated bran of the same moisture content, the numbers of *A. chevalieri* per gram of bran increased greatly between the sixth and twentieth day, and the free fatty acids similarly increased.

In the unautoclaved bran with 14.5% moisture, the bacteria had increased to 60 million per gram of bran after one day of storage and thereafter varied somewhat in number throughout the storage period. The types remained the same. Molds and filamentous yeasts had increased slightly by the second day and more by the thirteenth day, a yeast in the genus *Endomycopsis* becoming predominant. The rate of formation of free fatty acids was relatively steady until the thirteenth day, after which it increased. In the autoclaved-inoculated bran of the same moisture content, *A. chevalieri*, which on the third day showed a definite increase in numbers, grew only slowly thereafter. Free fatty acids in this sample showed a slight increase the third day and their rate of formation rose rapidly thereafter.

In the unautoclaved lot stored with 33.5% moisture content, the bacterial count was high (1.5 billion) on the first day, but by the sixth day it had increased to 4.5 billion. On the third day a change in the flora was noted, as indicated by changes in the types of colonies present. The count of molds and filamentous yeasts increased steadily through the sixth day, after which it decreased sharply. The *Endomycopsis* sp., referred to above, predominated on the first day of storage and

was the only filamentous organism found on the sixth day. Free fatty acids were formed throughout storage, but the rate of formation decreased steadily. Unfortunately for the purpose of the experiment, the autoclaved-inoculated lot remained free of bacteria only three days, and changed from 800 bacteria per gram of bran on the sixth day to 1,278,000 bacteria per gram on the thirteenth day. The *A. chevalieri* had increased in numbers by the third day but did not change after that. On the other hand, the content of free fatty acids remained unaltered for the first three days, but an increase was noted on the sixth day.

Discussion

That moisture content of the bran was a limiting factor in the growth of microorganisms was demonstrated by the fact that no appreciable growth was observed except in bran which had come into hydroscopic equilibrium, either by gradual absorption or by immediate addition, with relative humidities higher than 65.2%. Above this humidity, certain organisms seemed to have optima for growth when present in a mixed culture; for example, *A. chevalieri* grew best at 80.3% whereas bacteria grew best at 100%. A similar phenomenon of selection was noted by Bottomley *et al.* (4) in experiments on corn, when at 80% relative humidity *A. glaucus* or *A. flavus* predominated late in storage but at 100% *A. flavus*, *A. tamarii*, and *Fusarium* sp. were favored. In contrast to the mixed cultures, *A. chevalieri* thrived equally well at 100% as at 80.3% relative humidity when grown in pure culture in autoclaved bran. This effect of external and internal factors on a mixed microflora having synergistic or antagonistic relationships is discussed by Mossel and Westerdijk (18).

Moisture content was a limiting factor also in the formation of free fatty acids. The effects on unautoclaved and autoclaved bran differed. In unautoclaved bran free fatty acids formed at a rapid initial rate, especially at the higher moisture contents. Autoclaved bran showed no increase in free fatty acids regardless of moisture content as long as the sample remained sterile. In autoclaved bran which had been inoculated with *A. chevalieri*, a similar optimum moisture content was demonstrated for the production of free fatty acids as was shown for the growth of the mold in mixed culture.

Thus moisture had a similar effect upon both microorganisms and formation of free fatty acids and both often increased at the same time in the same sample. One example of this was the high initial rate of formation of free fatty acids with the marked increase in the number of bacteria in the unautoclaved bran stored at 100% relative humidity (Experiment Number 2). Another example in the same experiment was the excessive growth of *A. chevalieri* at 80.3% relative humidity

late in storage and the apparently simultaneous increase in production of free fatty acids.

The indication of a relationship between microorganisms and the production of free fatty acids is demonstrated more emphatically by the fact that inoculation of autoclaved bran with *A. chevalieri* (Experiment Number 4) caused the production of free fatty acids.

Although a relationship between the growth of some microorganisms and the formation of free fatty acids is indicated, it must also be taken into account that in unautoclaved bran (which contains enzymes from both the bran and the usual mixture of microflora), free fatty acids formed from the beginning of storage, whether or not an increase in the numbers of microorganisms could be demonstrated. Moreover, they formed more slowly in the autoclaved-inoculated bran when dependent only on the growth of *A. chevalieri* than in the unautoclaved bran. Even though it is understood that the large numbers of microorganisms present originally in the bran might be physiologically active and exert some influence without showing increases by plate count, the effect of enzymes from the bran itself cannot be ignored.

The results of the present work support or explain the observations of several other authors, and should have application in preventing deterioration of stored bran. They demonstrate that the rate of formation of free fatty acids is dependent upon moisture; heating and drying stabilize the bran; bran will absorb moisture at the higher relative humidities; and free fatty acids will not be formed in bran which has been sterilized and inactivated by autoclaving and is maintained in this condition.

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THE INFLUENCE OF DRIED WHEY ON CAKE QUALITY[1]

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ABSTRACT

Cakes containing 20 to 40% fat and 100% sugar were prepared with and and without the addition of 15% dried edible whey. The effect of whey was to give a more tender, soft, moist crumb, as well as improved flavor, browning and keeping quality. At all fat levels, cakes that had whey added were characteristically greater in volume, and were scored higher by judges for texture, tenderness, and flavor at both 24 and 72 hours after baking. Softness, as measured by the Baker Compressimeter, was also increased if whey was included in the formula.

Comparison of pairs of cakes of different fat and whey levels showed that a reduction in fat from 40 to 30%, 30 to 25%, or 25 to 20% could be compensated for by the addition of 15% edible whey to the cake of lower fat content.

Cakes made with dried whey were found to be superior to cakes containing non-fat milk solids in moistness and flavor.

Spray-dried whey is a product of good nutritive value now being used in a number of manufactured foods. Whey contains about half the total solids content of milk. The casein and fat are removed in the making of cheese but most of the sugars, soluble proteins, vitamins and minerals of milk are in the dried whey. The protein is known to be of high biological value. Thiamine and, especially, riboflavin are present in edible whey in important amounts. When whey is treated with anionic and cationic resins, the resulting product, demineralized whey, has some of the minerals and vitamins removed.

Edible whey in a dried or condensed form has been used in bakery products (1, 2, 3, 4, 5) but detailed comparisons have not been reported. Preliminary studies were begun in this laboratory in 1948, with demineralized whey. The cakes containing whey were very tender and moist and of good volume, grain, texture and flavor. When 66 to 75% whey (based on the weight of flour) was included in the formula, a brown thick crust was produced. The preliminary experiments indicated that 5 to 30% dried whey in the formula was a usable range of concentration. In further work, demineralized and dried edible whey were compared. A small panel of judges was unable to detect differ-

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ences in the flavor of yellow cakes when these two types of dried whey were included at a 35% level.

The preliminary studies suggested that a detailed study of the quality of whey cakes would be of value. Therefore, five series of cakes were tested. The premise in Series 1 was that since whey appeared to give more tender, moist cakes of good volume, a reduction of sugar, fat and eggs should be tried. In Series 2, the purpose was to compare whey and non-fat milk solids in cakes, the criteria being moistness and flavor as evaluated by a large panel of judges. Series 3, 4 and 5 were designed especially to study the effect of varying the fat and whey concentration in cakes to define a possible substitution ratio. The experiments on these three series included objective as well as organoleptic methods of study.

Procedure

Formulas and Materials. Two formulas were used for the five series of yellow cakes included in this report. Series 1 were made from the basic formula with modifications as indicated in the headings of the table of results. The formula with reduced eggs and shortening, called the modified formula, was used for cakes of Series 2, 3, 4 and 5, again with modifications as noted.

	Basic Formula	*Modified Formula*
	g.	g.
Flour	100	100
Sugar	100	100
Eggs (fresh)	60	40
Shortening	50	20 to 40 as indicated
Milk solids	8	None except when indicated
Dried whey	as indicated	0 to 30 as indicated
Water	85	85 or as indicated
Baking powder	3.4	4
Salt	1.5	2

The eggs were fresh whole eggs, the number for any replicate being blended and aliquots weighed out for all the cakes in that replicate. A household type vegetable shortening was used in all cakes except Series 4 and Replicates 2, 4 and 6 of Series 3 when a bakery-type high-ratio shortening replaced the household type. The commercial baking powder contained sodium aluminum sulfate and calcium acid phosphate as acid ingredients. For Series 1, the flour was of a general purpose family type; for all others, cake flour was used.

The spray dried whey had been prepared from sweet whey from Swiss and cheddar cheese. The approximate composition as furnished by the manufacturer, The Western Condensing Company of Appleton, Wisconsin, was on the dry weight basis, 13.3% total protein, 72.8% anhydrous lactose, 0.9% fat and 8.7% ash. The product specifications

of the manufacturer noted above for the demineralized whey used for Series 1 and part A of Series 2 were lactose as monohydrate, not less than 80%, protein not less than 11%, ash not more than 2%, fat not more than 1% and moisture not more than 4%. The non-fat milk solids was a spray-dried product manufactured especially for bakery use.

Mixing Methods. The cakes of Series 1 were made by the conventional method, the total creaming and mixing time at low speed being five minutes. The cakes from 220 g. batter were baked in round aluminum pans having a capacity of 485 ml. in an oven at 190°C. for 35 minutes. For all other cakes, the fat and three-fourths of the water were added to the mixed sifted dry ingredients. This mixture was beaten at low speed for one minute. Then, the eggs were added and mixing was continued for one-half minute. Finally, the remaining water was added and the batter was beaten for one-half minute more. One hundred and twenty grams of batter were weighed into round aluminum pans which had a capacity of 300 ml. The cakes were baked at 190°C. for approximately 25 minutes. For the cakes of Series 2, the baking was done in square 8 in. pans for 40 minutes. Often, two or three times the amount of batter indicated in the formula was mixed at one time and used to make several cakes that would be used for the number of tests to be made. All cakes were wrapped in wax paper after cooling and held at room temperature 24 hours or longer before making the various tests.

Scoring Panels. The quality of the cakes was scored after 24 hours by a group of four to six experienced judges. In all cases, coded or "blind" samples were used for judging. For cakes of Series 1, the score card used was:

Crust: thickness, tenderness, gloss, stickiness, color	15
Crumb color	5
Grain	20
Texture: softness, silkiness, moistness	20
Tenderness	15
Flavor	25

For Series 3, 4, and 5, crumb color and crust were not scored and, at the 72 hour period, grain was also omitted from the scoring. For Series 2, groups of 69 and 75 students ranked three cakes for flavor and moistness or scored the cakes and checked a list of descriptive terms.

Objective Tests. The following tests were made on all cakes of Series 3, 4, and 5:

Batter Viscosity—Viscosity measurements of the batters were made with a Brabender Viscosimeter using the pin paddle. The readings were expressed in arbitrary units of consistency (1-1000) which gave relative degrees of viscosity.

Weight and Volume—The volume of the cakes was measured by the seed displacement method. Three cakes from each variation were used and the average computed from the three for each replicate. Specific gravity was computed from the weight and volume data.

Compressibility—The compressibility of the cakes at various periods after baking was tested with a Baker Compressimeter. Samples of the cakes were prepared for this test by making cuts from top to

TABLE I

COMPARISON OF YELLOW CONTROL CAKES OF SERIES 1 WITH WHEY CAKES (35% DEMINERALIZED WHEY) OF VARIOUS FORMULAS

	Control Cakes (No Whey)	Whey Cakes with One-third Reduction in:		
		Sugar	Shortening	Egg
Crust	11.9	12.1	12.9	12.9
Texture: grain and silkiness	34.9	34.8	35.2	35.7
Tenderness	13.2	13.6	13.8	14.1
Crumb Color	5.0	4.9	4.9	5.0
Flavor	22.9	22.6	22.9	22.6
Total	87.9	88.0	89.7	90.9

	Control Cakes (No Whey)	Whey Cakes Dried Milk Omitted		
		No Reduction in:	One-third Reduction in:	
		Eggs, Sugar or Shortening	Egg and Shortening	Egg, Sugar, Shortening
Crust	13.5	13.3	13.3	11.2
Texture: grain and silkiness	34.8	35.2	33.5	33.0
Tenderness	13.8	13.8	13.9	13.5
Crumb Color	4.9	4.9	4.8	4.9
Flavor	23.8	23.2	23.5	23.0
Total	90.8	90.4	89.8	86.6

bottom of the cake with a cutter that had the same diameter as the plunger of the compressimeter (1½ inches). These were then cut into sections ⅝" high; this height allowed the sample to be placed on the platform of the compressimeter under the plunger with the indicators resting on zero. Readings were made of both load and deformation after the motor had been revolving for 10 seconds. The compressibility ratio was computed by dividing the deformation by the load. Thus, a high ratio indicates greater compressibility.

Results and Discussion

Series 1: *The effect of reduction of enrichening ingredients when whey was added.*

Since the preliminary work on whey cakes indicated greater moistness and tenderness, reductions in other enrichening ingredients were tried in Series 1 and recorded in Table I. With reductions of one third shortening, sugar, or eggs in the yellow cake formula, scores for the demineralized whey cakes still compared favorably with those of the control cakes. When both eggs and shortening in the whey cakes were reduced, they scored about as high as the control cakes. A reduction of eggs and shortening is of advantage from the standpoint of cost. This influence of whey on moistness and tenderness was studied in greater detail in later experiments of Series 3, 4 and 5.

Series 2: *Comparison of dried whey and non-fat milk solids in cakes.*

In two experiments involving larger numbers of judges, the flavor of cakes made with whey was compared with that of control cakes, and with cakes containing non-fat milk solids. In the first test, the pro-

TABLE II

Comparison of the Flavor and Moistness of Yellow Cakes

Part A	Cake A (35% Demineralized Whey)	Cake B (16% Non-fat Dry Milk Solids)	Cake C (Fresh Milk, 93%)
Average flavor score (based on 10)	6.7	6.3	7.3
Number of students scoring cake highest	25	19	25
Number of students checking the following descriptive terms:			
Stale	4	14	11
Too sweet	9	4	7
Not sweet enough	9	9	13
Too much egg	2	3	0
Buttery	17	7	5
Excellent	7	9	12
Desirable	24	17	23
Peculiar flavor	7	10	3
Lack of flavor	1	2	4

Part B	Cake D (15% Dried Whey)	Cake E (15% Non-fat Dry Milk Solids)	Cake F (7.5% Non-fat Dry Milk Solids)
Number of students ranking cake as:			
Best flavor	33	14	28
Intermediate in flavor	28	16	31
Least desirable	14	45	16
Number of students ranking cake as:			
Most moist	52	4	19
Intermediate in moistness	19	18	39
Most dry	4	53	18

portions of whey solids and milk solids were high in order to elicit definite indications of flavor differences. In both Cakes A and B, the water was increased to 105 parts rather than 85. (The results on the 24 hour old cakes are summarized in Table II, Part A.) A slight preference was shown for the cakes made with fluid milk as indicated by the average score. However, the whey cake and fluid milk cake

TABLE III

QUALITY CHARACTERISTICS OF CAKES CONTAINING 30 OR 40% FAT AND DIFFERENT AMOUNTS OF WHEY (6 REPLICATIONS OF SERIES 3)

	Fat—40%			Fat—30%		
	Whey—none	10% Whey	15% Whey	Whey—none	10% Whey	15% Whey
Batter viscosity Average	566	652	757	374	457	509
Volume (ml.) Average	301	309	317	311	313	316
Compressibility Ratio						
3 hours[1]	0.51	0.60	0.64	0.54	0.63	0.62
24 hours	0.37	0.43	0.52	0.35	0.41	0.43
72 hours	0.32	0.33	0.38	0.25	0.31	0.35
120 hours	0.20	0.22	0.27	0.17	0.24	0.25
Judges' Average Scores of Cakes 24 Hours After Baking						
Grain	15.9	16.0	15.6	13.9	13.9	14.2
Texture	16.1	16.8	17.2	14.3	15.6	16.1
Tenderness	12.8	12.8	13.2	11.7	12.2	12.8
Flavor	18.6	18.8	19.6	18.3	19.0	19.4
Sum of scores[2]	63.4	64.4	65.6	58.2	60.7	62.5
Judges' Average Scores 72 Hours After Baking						
Texture	14.2	14.2	15.6	12.4	14.0	13.7
Tenderness	11.2	12.2	12.8	10.5	11.4	11.6
Flavor	19.0	19.4	20.1	18.1	18.9	18.9
Sum of scores[2]	44.4	45.8	48.5	41.0	44.3	43.2

[1] Only two replications at the 3 hour period in this series.
[2] Maximum scores in all characteristics would give a total of 80 at 24 hours and 60 at 72 hours storage period.

were scored highest by the same number of students. More of the judges considered the flavor of the dried and fresh milk cakes "stale" and the whey cake "buttery."

In the second test (Table II, Part B), samples of three cakes 48 hours old were ranked for moistness and flavor by a class of 75 students. The cake which contained 15% edible whey (Cake D) was rated best

in flavor by 33 of the 75 students, least desirable by 14, and intermediate by 28. In contrast, the cake containing 15% non-fat milk solids (Cake E) was ranked best in flavor by only 14 students, least desirable by 45, and intermediate by 16. In flavor, the judges ranked Cake F containing 7.5% milk solids slightly lower than Cake D containing 15% dried whey.

In moistness, the judges indicated a marked preference for whey in contrast to milk solids as shown in Part B of Table II. It is to be noted that as many judges ranked the cake made with 15% milk solids most dry as rated the cake made with 15% whey the most moist. The cake made with 7.5% milk solids was intermediate. Thus, whey, whether demineralized or not had no deterimental effect on flavor. An outstanding value of the addition of whey shown in these cakes was the degree of moistness retained in cakes held in storage 48 hours.

TABLE IV

ANALYSES OF VARIANCE OF JUDGES TEXTURE SCORES AND OF THE COMPRESSIBILITY RATIOS OF CAKES OF SERIES 3

Source of Variation	Degrees of Freedom	Mean Squares for Average Texture Scores	Mean Squares for Average Compressibility Ratios
Replications	5	1.34 N.S.	0.003 N.S.
Formulas			
Fat	1	19.36**	.012*
Whey	2	5.40*	.044**
Interaction	2	0.69 N.S.	.006 N.S.
Error	25	1.05	0.002
Total	35		

Series 3: *Detailed study of the effect of various amounts of whey on cakes containing 30 or 40% fat.*

The data from Series 3 cakes are presented in Table III. There are to be noted three facts: the increases in batter viscosity when either fat or whey content was increased, the increase in volume when the amount of whey was increased in the cakes with 40% fat and the marked increase in compressibility of the cakes containing 10 or 15% whey over that of the cakes containing no whey at either fat level.

In judges' scores, presented also in Table III, grain was found to be improved by the higher fat content but not by whey additions, whereas texture and tenderness were enhanced by both factors. The cakes with the higher fat content were of consistently finer, more uniform grain, than those of lower fat content. The grain of the cakes with 40% fat was, however, different in that with no whey, it was more compact in contrast to the cakes with 10 or 15% whey which were more

porous and feathery. In comparison, the cakes of 30% fat were, in general, more coarse and had a more uneven, open grain. Judges scores for texture, which included silkiness, softness and moistness, were consistently higher for the cakes of 40% fat. But within each fat level, the scores were higher in proportion to the amount of whey added. The scores for tenderness revealed a greater increase from

TABLE V

QUALITY CHARACTERISTICS OF CAKES HAVING LOW FAT LEVELS WITH OR WITHOUT WHEY (6 REPLICATIONS OF SERIES 4)

	No Whey			Whey—15%		
	20% Fat	25% Fat	30% Fat	20% Fat	25% Fat	30% Fat
Batter viscosity Average	522	578	775	602	687	842
Volume of cake (ml.) Average	307	304	310	323	319	322
Specific gravity of cakes Average	0.34	0.34	0.33	0.32	0.32	0.31
Compressibility Ratio						
3 hours	0.42	0.38	0.44	0.50	0.49	0.51
24 hours	0.40	0.35	0.38	0.45	0.45	0.42
72 hours	0.32	0.29	0.31	0.35	0.36	0.32
120 hours	0.22	0.22	0.23	0.28	0.27	0.28
Judges' Average Scores of Cakes, 24 Hours After Baking						
Grain	13.1	15.8	17.1	14.1	16.1	16.2
Texture	14.2	15.4	16.6	15.5	17.0	17.3
Tenderness	11.2	11.8	13.0	12.3	13.4	13.9
Flavor	17.8	18.0	19.5	19.0	19.8	20.2
Sum of scores[1]	56.3	61.0	66.2	60.9	66.3	67.6
Judges' Average Scores of Cakes, 72 Hours After Baking						
Texture	11.7	12.7	14.4	12.7	13.9	15.2
Tenderness	10.8	11.8	12.8	12.1	12.8	13.4
Flavor	17.2	18.4	19.2	18.3	18.3	19.2
Sum of scores[1]	39.7	42.9	46.4	43.1	45.0	47.8

[1] Maximum scores in all characteristics would give a total of 80 at the 24 hours and 60 at 72 hours storage period.

whey additions in the cakes of 30% than in the 40% fat cakes. The addition of whey produced a small but definite trend toward higher flavor scores. Occasionally a judge commented that the cake with 30% fat and no whey was "flat" in flavor. Most of these observations on the cakes 24 hours after baking were substantiated by the scoring after 72 hours storage, although, for certain characteristics, the 0 and

10% whey cakes were alike and, for others, the 10 and 15% whey additions gave similar results.

Since average texture scores and compressibility ratios in Table III showed the greatest influence of whey additions, these data for the 24 hour old cakes were subjected to statistical analysis. The analyses of variance given in Table IV showed a highly significant increase in compressibility and a significant improvement of texture due to whey addition. Fat also produced a highly significant improvement of texture and a significant increase in compressibility.

TABLE VI

ANALYSES OF VARIANCE OF TEXTURE SCORES AND COMPRESSIBILITY RATIOS OF CAKES OF SERIES 4 AFTER 24 HOURS STORAGE

Characteristic Tested	Source of Variation	Degrees of Freedom	Mean Square
Judges' scores on texture	Replicates	5	16.02
	Treatments	5	54.39
	Fats	2	86.76**
	Wheys	1	89.61**
	Interaction	2	4.42 N.S.
	Judges	4	5.94
	Replicates × treatment	25	6.41**
	Judges × replicates[1]	19	12.03**
	Judges × treatment	20	3.21 N.S.
	Residual	95	2.86
	Total	173	
Compressibility ratios	Replicates	5	0.068**
	Treatments	5	0.008**
	Fats	2	0.002 N.S.
	Wheys	1	0.032**
	Interaction	2	0.002 N.S.
	Error	25	0.001
	Total	35	

[1] Since the interaction of judges × replicates was highly significant, this mean square rather than residual mean square was used as the "error" term to test significance of all treatment effects.

Series 4: *The effect of 15% whey on cakes of 20, 25, or 30% fat content.*

The summary of data for Series 4 is given in Table V with detailed statistical analyses in Table VI and VII. Individual rather than averages of judges' scores of texture and compressibility ratios for the six replicates were used so that partition of variances could be made. Statistically significant effect on compressibility of whey but not of fat content was shown on the cakes 24 hours old. The data on texture and compressibility of the cakes 72 hours old were also subjected to analysis of variance but for the sake of brevity, details are not presented. For the compressibility ratios of these 3-day old cakes, the

interaction of "fats" and "wheys" was statistically significant and the degrees of freedom too small to make a test of significance. Both "fats" and "wheys" showed a significant improvement in texture at both the 24 and 72 hour storage times. The addition of 15% whey,

TABLE VII

ANALYSES OF VARIANCE OF JUDGES SCORES ON CAKES OF SERIES 4 AFTER 24 HOURS STORAGE

Characteristic Tested	Source of Variation	Degrees of Freedom	Mean Square
Judges' scores on tenderness	Replicates	5	5.18
	Treatments	5	36.90
	Fats	2	47.94**
	Wheys	1	86.81**
	Interaction	2	0.91 N.S.
	Judges	4	12.33
	Replicates × treatments	24	1.83 N.S.
	Judges × replicates[1]	19	4.16**
	Judges × treatments	20	1.51 N.S.
	Residual	95	1.30
	Total	173	
Judges scores on grain	Replicates	5	26.57
	Treatments	5	73.27
	Fats	2	167.32**
	Wheys	1	5.69 N.S.
	Interaction	2	13.01 N.S.
	Judges	4	93.50
	Replicates × treatments	25	10.48**
	Judges × replicates[1]	19	10.83**
	Judges × treatments	20	6.72 N.S.
	Residual	95	4.09
	Total	173	
Judges scores on flavor	Replicates × judges	32	52.32
	Replicates	5	19.11**
	Judges within replicates	27	58.47**
	Treatments	5	30.42
	Fats	2	30.56**
	Wheys	1	82.75**
	Interaction	2	4.13 N.S.
	Residual	160	5.20
	Total	197	

[1] Since the interaction of judges × replicates was highly significant, this mean square rather than residual mean square was used as the "error" term to test significance of all treatment effects.

thus, accounts for an improvement in texture and a marked increase in compressibility of these cakes containing 20, 25, 30% high ratio fat.

As in Series 3, the grain was not consistently changed by whey additions but the fat content exerted a significant influence. In each series, the concentration of both fat and of whey affected the tenderness scores to a significant degree at both the 24-hour and 72-hour storage

periods. In these cakes of low fat content, the judges scores for flavor showed a significant improvement when fat was increased or whey included in the cakes stored for 24 hours. After the 72-hour storage period, the cakes were judged to be statistically different when fat content was varied but not for additions of a whey. The statistical analyses in Tables VI and VII showed that, beyond the variation from judge to judge and from replicate to replicate and their interactions, a significant improvement in tenderness and texture and an increase in compressibility at 24 hours resulted from the addition of whey to cake formulas containing from 20 to 40% fat.

Correlation of Texture Scores and Compressibility Ratios. It was of interest, in this study, to correlate the subjective measure of texture

TABLE VIII

JUDGES SCORES ON "WHEY" CAKES OF COMPARABLE BATTER VISCOSITY
(Averages of 3 replications; all cakes made with 30 per cent fat)

	Per Cent of Whey			
	0	10	15	30
Cakes, 24 hours after baking:				
Grain	16.8	16.8	15.5	15.5
Texture	15.4	17.1	16.9	18.5
Tenderness	12.7	13.5	13.2	14.2
Flavor	20.4	20.4	20.6	21.2
Sum of scores[1]	65.3	67.8	66.2	69.3
Cakes, 72 hours after baking:				
Texture	14.1	14.6	14.8	16.9
Tenderness	11.4	12.9	12.6	13.0
Flavor	18.6	20.4	19.4	20.0
Sum of scores[1]	44.1	47.9	46.8	49.9

[1] Maximum scores in all characteristics would give a total of 80 at 24 hours and 60 at 72 hours storage period.

by the judges with the compressibility as tested objectively. Whereas the judging panel considers such characteristics as silkiness or harshness, moistness or dryness, softness or firmness in assigning a texture score, the Baker Compressimeter measures softness only. For Series 3, the linear correlation coefficient, between mean texture scores and compressibility ratios was +0.578 which, for 34 degrees of freedom, is significant at the 1% level. For Series 4, $r = +0.386$ which is significant at the 5% level. These computations were made from the mean squares in Tables IV and VI.

Series 5: *Effect on cakes of increasing whey content with adjustment of batter viscosity by added water.*

This series was designed to learn whether the influence of the whey on batter viscosity was, in some manner, leading to false conclusions regarding the cake qualities induced by adding whey to cake formulas. The data of Tables III and V, show that whenever whey or fat was increased in the formula, the cake batter was more viscous. In contrast to an increase in viscosity caused by added flour, both fat and whey give increased tenderness. The addition of whey resulted in a greater compressibility and higher texture scores. Accordingly, the cakes of Series 5 were made with 30% fat and a whey content of 0, 10, 15 or 30%. The water content was varied from 85 to 90%.

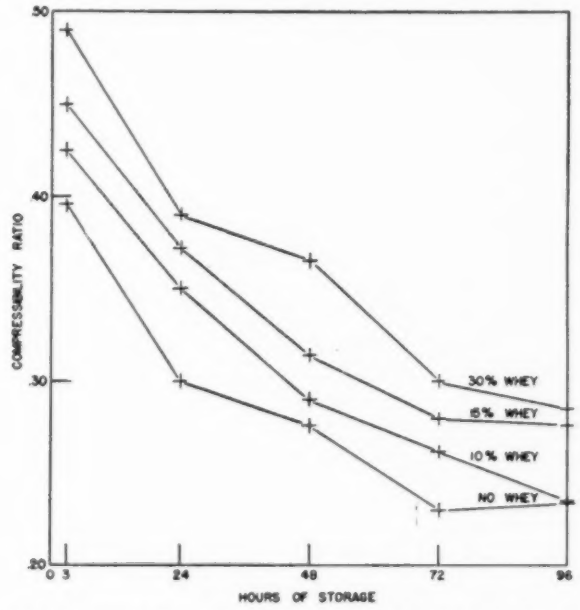

FIG. 1. Changes in the compressibility of the cakes of series 5 during storage.

Although attempts to obtain uniform batter viscosities were not completely successful, the viscosities were brought within a close range of each other. The final average batter viscosities were 700, 690, 683 and 693 for the batters containing 0, 10, 15 and 30% whey. The weights of all the cakes after baking were similar; the average loss in baking was about 16% as had been true for Series 3 and 4 as well. There was little variation in volume, the average volumes being 313, 317, 317 and 315 cc. respectively for the cakes containing 0, 10, 15 and 30% whey.

There were marked differences in the compressibility of the cakes of the four different formulas at each of the storage periods tested. These are presented graphically in Fig. 1. The shape of the curves shows clearly that the rate of change in compressibility was similar for the cakes made with varying amounts of whey. However, there was a marked difference among formulas in the softness and compressibility at any time after the cakes were baked and in direct proportion to the amount of whey in the formula. The softness of the cakes increased with larger additions of whey to the point where the cakes with 30%

TABLE IX

SIMILARITY OF THE QUALITY OF THREE PAIRS OF CAKES OF DIFFERENT FAT AND WHEY CONTENT

	Pair 1		Pair 2		Pair 3	
	40% Fat No Whey	30% Fat 15% Whey	30% Fat No Whey	25% Fat 15% Whey	25% Fat No Whey	20% Fat 15% Whey
Average volume	301	316	310	319	304	323
Compressibility ratios						
3 hours	0.51	0.62	0.44	0.49	0.38	0.50
24 hours	0.37	0.43	0.38	0.45	0.35	0.45
72 hours	0.32	0.35	0.31	0.36	0.29	0.35
120 hours	0.20	0.25	0.23	0.27	0.22	0.28
Judges scores—24 hours after baking						
Grain	15.9	14.2	17.1	16.1	15.8	14.1
Texture	16.1	16.1	16.6	17.0	15.4	15.5
Tenderness	12.8	12.8	13.0	13.4	11.8	12.3
Flavor	18.6	19.4	19.5	19.8	18.0	19.0
Sum of scores	63.4	62.5	66.2	66.3	61.0	60.9
Judges scores—72 hours after baking						
Texture	14.2	13.7	14.4	13.9	12.7	12.7
Tenderness	11.2	11.6	12.8	12.8	11.8	12.1
Flavor	19.0	18.9	19.2	18.3	18.4	18.3
Sum of scores	44.4	43.2	46.4	45.0	42.9	43.1

whey seemed excessively soft and crumbly. Hence, in this series, the addition of whey produced cakes of greater compressibility even when batter viscosity, volume and weight of baked cake were similar. It appears that the effect of whey in giving tender moist cakes is a characteristic of whey *per se*. Whether the increase in viscosity due to whey addition was adjusted in Series 5 or not as in Series 3 and 4, better texture and tenderness resulted.

Substitution Ratios of Fat and Whey. One of the purposes of this study was to learn if a reduction in fat in the cake formula could be

compensated for by the addition of dried whey. Table IX shows a comparison of three pairs of cakes from Series 3 and 4 in which a cake of higher fat content is compared with one that had lower fat and the addition of 15% whey. In general, the cakes of lower fat with whey added received equally high scores for texture, tenderness, and flavor as those of higher fat but with no whey added. However, increases in fat did produce cakes of finer grain with a more compact structure. Thus, the cakes were not entirely similar, but the judges considered them equally acceptable in a summation of quality characteristics. The cakes with lowered fat and 15% whey had the additional advantage of greater volume, and consistently softer texture, as measured by the Baker Compressimeter, at all times from three hours to 120 hours after baking. Thus, a reduction in fat from 40 to 30% or from 30 to 25% or from 25 to 20% fat can be compensated for by the addition of whey to give cakes of good quality.

Another consideration would be that of a change in nutritive value when there was a reduction of shortening and eggs, and whey substituted for milk. A computation was made for cakes of the basic formula which contained 60% eggs and 50% fat, and for those of the modified formula containing 15% whey, no milk, and a one third reduction in fat and eggs. There is no difference in caloric value, but a slight reduction in protein and vitamin A content, and an increase in thiamine and riboflavin in the whey cake.

The formula used for these cakes was one of comparatively low (100%) sugar content. On the basis of preliminary work, it is considered impossible to obtain the marked improvement shown by whey addition in cakes of such low fat contents as in these series merely by adding a greater amount of sugar. However, comparative studies are in progress to learn the qualities in cakes induced by increased sugar, mixtures of sugars and of sugar with whey.

Acknowledgments

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THE DECREASE IN SUSCEPTIBILITY OF BREAD CRUMB STARCH TO BETA-AMYLASE DURING STALING[1, 2]

S. S. JACKEL, A. S. SCHULTZ, F. D. SCHOONOVER, and W. E. SCHAEDER[3]

ABSTRACT

An improved fermentation method was used to study changes in the susceptibility of bread crumb starch to attack by beta-amylase during the process of bread staling. The amount of attackable starch and the rate of saccharification decreased with increasing staleness of the bread. The changes were rapid at low temperatures, but appreciably less so at 30°C. Thus the change in the starch of the bread was two-thirds complete after 17 hours storage at 8°C. but only one-third complete after storage for the same period at 30°C. Polyoxyethylene monostearate emulsifiers did not retard development of these changes appreciably. Measurement of the susceptibility of bread crumb starch to enzymatic attack may be carried out by simple procedures which are useful in the evaluation of bread staling.

Bread staling is a complex phenomenon, the mechanism of which is not clearly understood. The literature indicating that staling is accompanied by changes in taste and aroma, by increased crumb opacity, hardness, crumbliness and starch crystallinity, by decreases in soluble starch content and absorptive capacity of bread crumb, and by decreased susceptibility of bread crumb to attack by flour diastase was reviewed recently by Geddes and Bice (6). Considerable evidence pointing towards the important role of starch retrogradation in the staling of bread has accumulated and has also been recently reviewed (9, 22). Katz (7) concluded that retrogradation of starch was the prominent factor bringing about staling and Schoch and French (15) reported that it was the amylopectin fraction of starch which was involved in staling retrogradation. Schultz and Landis (19) and Katz (8) established that retrograded starch in stale bread was less susceptible to attack by flour diastase than the gelatinized starch in freshly baked bread, confirming the previous work on starch pastes by Maquenne (12) and Sallinger (14).

An improved fermentation method for measuring the susceptibility of the starch of bread crumb to attack by beta-amylase was developed in the present studies and was employed in following the changes in

[1] Manuscript received December 21, 1951.
[2] A report based on a portion of the data given herein was presented before the Thirty-Seventh Annual Meeting of The American Association of Cereal Chemists, Dallas, Texas, April, 1952.
[3] The Fleischmann Laboratories, Standard Brands Inc., New York 51, New York.

starch susceptibility during the staling of bread stored at 8°C. and 30°C. The effect of polyoxyethylene monostearate emulsifiers on the rate of these changes was investigated.

Materials and Methods

Bread. White bread was prepared on a commercial scale in 50 lb. flour batches by the sponge dough process. Regular bakery plant equipment, involving high speed mixing, dividing, rounding, intermediate proofing and moulding (automatic make-up), was used and commercial schedules were maintained. Sixty per cent of Northwestern flour was used in the sponge stage and 40% of Southwestern flour in the dough stage. Control loaves were baked by the basic formula given below. Experimental loaves designated "no fat" were baked with omission of shortening. Experimental loaves designated "emulsifier" were baked by the basic formula with addition of one-half per cent (based on flour) of emulsifier (polyoxyethylene monostearate type) to the dough stage. The basic bread formula and conditions of baking were:

Sponge Stage	Ingredients	Dough Stage	Percentage on Flour
30 lb.	Flour	20 lb.	100
18 lb.	Water	15 lb.	66
1 lb.	Bakers' compressed yeast	—	2
3 oz.	Mineral yeast food and dough improver containing potassium bromate	—	0.375
8 oz.	Diastatic malt extract, 20°L.	—	1
	Vitamin Enrichment Wafer	—	—
—	Salt	1 lb.	2
—	Sugar (cane)	3 lb.	6
—	Non fat milk solids	1.5 lb.	3
—	Shortening (hydrogenated)	1.5 lb.	3

77°F. (25°C.) sponge temperature from mixer. 4 hours sponge fermentation time. 80°F. (26.7°C.) dough temperature from mixer. Floor time—15 minutes. Proof time—65 minutes.

After the baked loaves reached room temperature they were sealed in moisture proof cellophane and stored at 30°C. and 8°C. for future testing. The loaves were evaluated for degree of freshness initially and at 17, 41, 65 and 89 hours after baking by the fermentation method using the criterion of starch susceptibility to beta-amylase action. Subjective softness was determined on the entire loaf of bread by "squeeze" and on the sliced bread by taste and by feel.

Fermentation Method. The fermentation method described by Schultz and Landis (18) for the study of diastatic action was applied by those authors to a study of starch retrogradation in bread staling (19). The method, with some further study and modification, was applied to the present investigation. The details in brief are:

Twenty grams of interior bread crumb were weighed into a 250 ml. wide mouth reaction bottle and 100 ml. of water at about 25°C. added. The wetted crumb was dispersed by stirring for one minute with an electric stirrer [Hamilton-Beach] equipped with a button type impellor. With this stirrer identical final results were obtained when the stirring period was varied from 30 seconds to 5 minutes. The temperature of the suspension did not increase by more than one degree during the mixing period. Ten milliliters of potassium citrate buffer,[4] 2 ml. of thiamine hydrochloride solution (10 μg./ml.), and 25 ml. of a 20% suspension of compressed bakers' yeast were added to each bottle. The bottles were stoppered at once, transferred to the shaking device in the 30°C. constant temperature water bath of the Fleischmann Fermentometer (18), and connected to the 500 ml. gasometers for measurement of carbon dioxide evolution. The small scale Fleischmann Aminco Fermentometer (17) may be used if all quantities are reduced by half.

Conversion of the starch attackable by beta-amylase was initiated when essentially no fermentable sugar remained, as indicated by production of less than 3 ml. of gas in a fifteen minute period. This was accomplished by opening the reaction bottles, resetting the gasometers, and adding 35 mg. of commercial 2000° Lintner beta-amylase[5] to each bottle. The bottles were restoppered at once and the carbon dioxide evolved during the fermentation was collected and measured at convenient intervals. The total gas evolution, after initiation of conversion by beta-amylase addition, bears a stoichiometrical relationship to the quantity of starch susceptible to attack by beta-amylase. The amount of gas produced in two hours may be taken as a measure of the amount of susceptible starch and hence indicative of the state of freshness of the crumb sample.

Results and Discussion

The availability and self-evident advantages of water soluble, purified and standardized beta-amylase preparations recommended their use in the fermentation method in place of the variable and insoluble non-diastated flour originally used by Schultz and Landis (19). The effect of beta-amylase levels over the range of 10 to 90° Lintner (representing the addition of 5 to 45 mg. of 2000° Lintner beta-amylase) on saccharification of bread crumb starch is shown in Fig. 1. The bread used in this study was baked by the basic formula and stored in moisture proof cellophane for 41 hours at 30°C.

[4] 100 g. of potassium citrate, $K_3C_6H_5O_7 \cdot H_2O$, 20 g. of citric acid $H_3C_6H_5O_7 \cdot H_2O$, and 20 g. of ammonium acid phosphate, $NH_4H_2PO_4$, per liter of solution. pH 5.2.

[5] Wallerstein Laboratories (New York, N. Y.) analytical grade beta-amylase was used. Specifications for this product have been published (1). The beta-amylase was added conveniently as 10 ml. of a freshly prepared aqueous solution.

Effect of Beta-amylase Concentration on Rate of Saccharification. The quantities of gas produced and the rates of gas production were almost identical when beta-amylase activities of 60, 70 or 80° Lintner were employed. At the 10 and 30° Lintner levels the relationship between gas produced and time of fermentation was found to be practically linear during the first few hours. The rate of reaction however, in view of the data at higher enzyme levels, was limited by the rate of saccharification of the starch. At the higher enzyme levels the limiting factor during the initial part of the fermentation appeared to be the ability of the yeast to ferment the enzymatically-produced maltose.

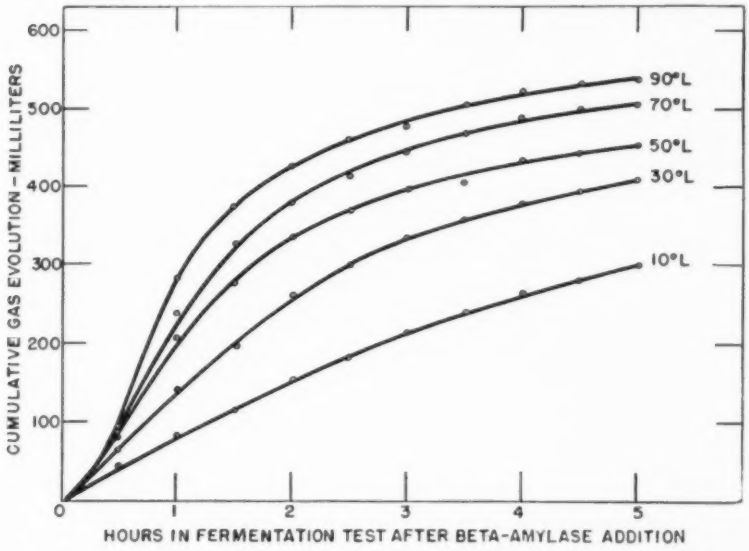

FIG. 1. Effect of beta-amylase concentration on carbon dioxide evolution in fermentation test.

By the end of the first hour the yeast was well acclimatized to maltose and the rate then was limited only by the amount of starch present that was attackable by beta-amylase. In view of these considerations the conditions of the analytical method were set at a beta-amylase level of 70° Lintner (35 mg. of 2000° Lintner material) for conversion and a fermentation time of two hours after initiation of conversion.

Effect of Storage Temperature and Time on Starch Susceptibility. The data of Fig. 2 represent the behavior of bread (baked by the basic formula) in the fermentation test after storage at 8° and 30°C. for various lengths of time. It is evident from these data that bread staling is accompanied by changes, presumably retrogradation of the

starch, which reduce the amount of starch attackable by beta-amylase and which are reflected in the fermentation test by large differences in gas evolution. In agreement with earlier data (5, 13, 19), it may be concluded that the rate of staling, as measured both subjectively and by the fermentation test, is influenced by the temperature of storage. Bread stored for 41 hours at 30°C. was appreciably fresher than similar samples stored at 8°C. for 17 hours. Gas production after 65 hours of storage at 30°C. was approximately equivalent to that observed with the loaves stored at 8°C. for 17 hours. It is noteworthy that the rate of retrogradation of starch pastes has been reported to show similar

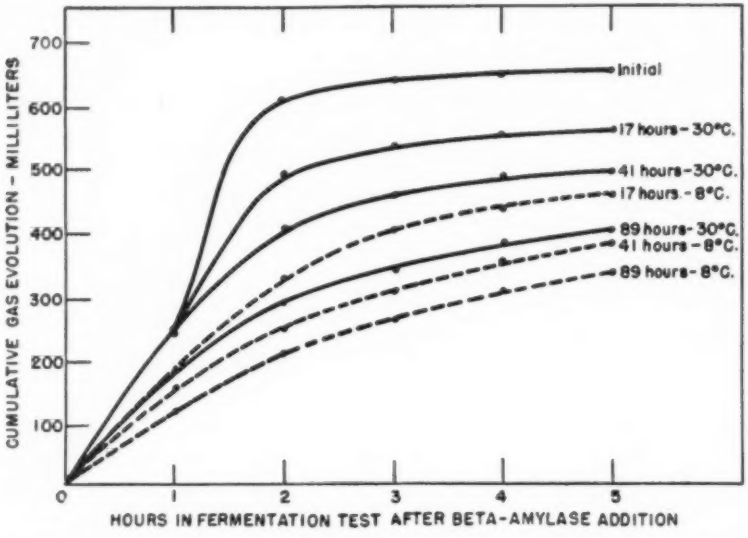

FIG. 2. Effect of the time and temperature of storage of bread crumb on amount and rate of gas evolution in fermentation test.

temperature dependence (10, 12), the rate increasing rapidly as the temperature is lowered.

Staling has been reported to be a rapid process (6). Measurements of changes in crumb susceptibility to flour diastase by Schultz and Landis (19), in compressibility by Platt (13), and in water abosrption in the Farinograph by Fuller (5) demonstrated that the greatest amount of staling, at storage temperatures of 2 to 21°C., occurred within the first 25 hours after baking. The data for Table I for the bread stored at 8°C. are in general agreement with these reports; approximately 65% of the decrease in gas evolution which took place during 89 hours of storage had occurred within the first 17 hours. At 30°C., however,

the rate of change in crumb susceptibility to beta-amylase was decreased appreciably, only one-third of the 89 hour decrease in gas evolution taking place within the first 17 hour period. It should be pointed out that commercial bread has already undergone a portion of its staling when it reaches the consumer. This bread is considered fresh and hence, from the consumer's point of view, the important part of staling is that occurring after this initial period.

Effect of Polyoxyethylene Monostearate Emulsifiers on Starch Susceptibility. Emulsifiers of the polyoxyethylene monostearate type have been reported (4) to preserve the softness of bread crumb and to retard development of other characteristics of staling. Bradley (2) and

TABLE I

EFFECT OF STORAGE ON RATE OF STALING OF BREAD[1]

Storage Conditions	Bread Baked by Basic Formula		Bread Baked Without Fat	
	Gas Evolution	Decrease in Gas Evolution	Gas Evolution	Decrease in Gas Evolution
	ml.	%	*ml.*	%
Initial—oven fresh	605	—	586	—
30°C. storage—17 hours	487	37.6	486	31.2
41	406	63.4	394	59.9
65	342	83.8	338	77.3
89	291	100.0	265	100.0
8°C. storage—17 hours	331	67.0	329	66.2
41	252	86.3	236	90.2
65	196	100.0	210	96.9
89	196	100.0	198	100.0

[1] The data reported are the amounts of gas produced from 20 g. of bread crumb in two hours after conversion with 35 mg. of 2000° Lintner beta-amylase.

Skovholt and Dowdle (20) have recently called attention to the controversy as to whether emulsifiers are merely bread softeners or whether they retard the rate of firmness development. Carson, Marnett and Selman (3) examined the staling of bread baked with and without the addition of polyoxyethylene monostearate by various physical measurements. The bread baked with emulsifier staled less quickly than control bread by the criterion of compressibility changes but at an equal or faster rate by the criterion of changes in crumbliness. The authors concluded that of the traditional staling rate measurements only those based on changes in compressibility were of value. Bradley (2), however, concluded from similar data, including crumbliness, compressibility and subjective taste and feel measurements, that these emulsifiers

functioned only as bread softeners without actually inhibiting bread staling.

The data in Fig. 3 indicate that the bread containing polyoxyethylene monostearate produced appreciably less gas initially in the fermentation test than did the control bread not containing emulsifier. Favor and Johnston (4) and Lord (11) have demonstrated that polyoxyethylene monostearate emulsifiers increase the temperature at which starch begins to swell (gelatinization temperature) and also restrict the extent of swelling at higher temperatures. It appears likely from these considerations that the decreased gas production of the oven-fresh loaves

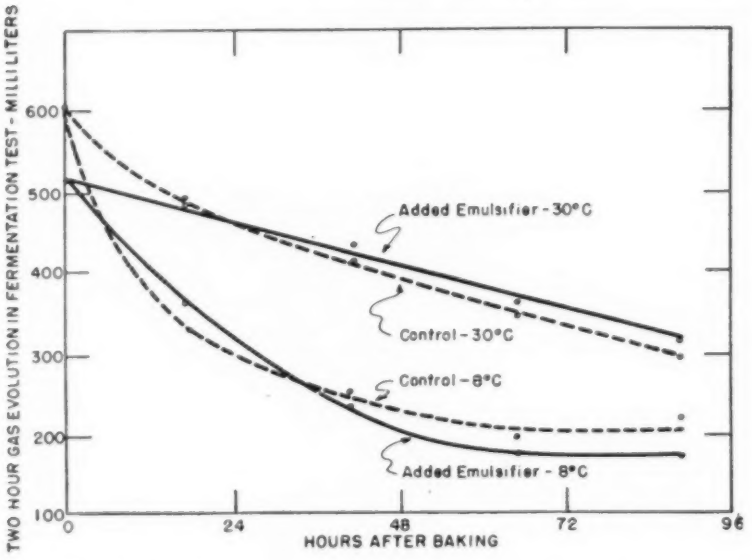

FIG. 3. Effect of polyoxyethylene monostearate and of time and temperature of storage of bread on amount of starch attackable by beta-amylase.

containing emulsifier is indicative of incomplete gelatinization of the starch. This is in agreement with the opinion stated by Lord (11) that polyoxyethylene monostearate emulsifiers produce the effect on starch of being partially stale even when freshly cooked. The data indicate further that although the amount of starch susceptible to beta-amylase decreased as the bread continued to stale differences between the control and emulsifier breads were relatively small beyond the initial period of 5 hours at 8°C. or 23 hours at 30°C.

The apparent lack of effect of the polyoxyethylene monostearate in retarding the development of decreased crumb susceptibility to beta-

amylase during staling of the bread was unexpected. Studies on autoclaved starch solutions, such as those of Lord (11), have indicated that polyoxyethlene monostearate emulsifiers are effective in reducing starch retrogradation by forming insoluble complexes with the amylose molecules which are then incapable of associating. It is likely that this phenomenon also occurs in bread but that it is of relatively small magnitude because of the small amounts of emulsifier that can be used practically. Schoch (16) has estimated that polyoxyethylene monostearate complexes 4 to 5 times its weight of linear starch fraction and that 0.5% (based on flour) of this emulsifier would result in only a

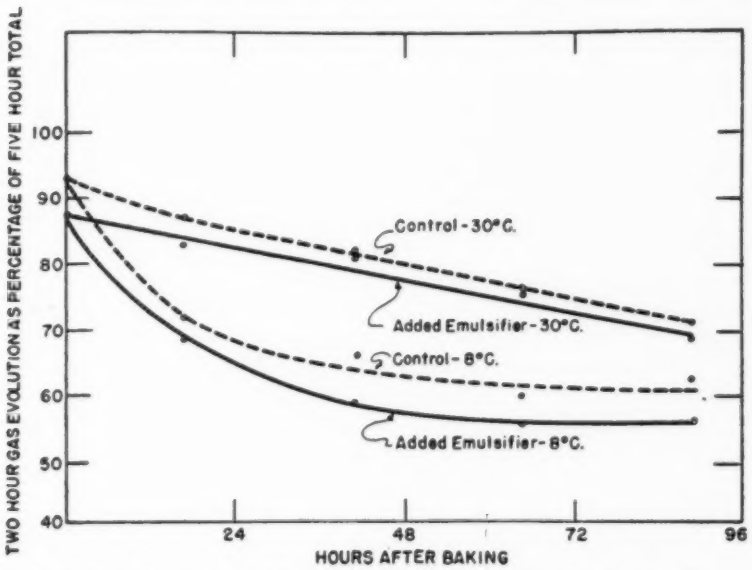

FIG. 4. Effect of polyoxyethylene monostearate and of time and temperature of storage on the attackability of starch by beta-amylase.

2% decrease in available starch. It would thus appear that the use of emulsifier at commercial levels does not influence the rate of attack by beta-amylase because of the small amount of substrate involved in complexing by the emulsifier.

It appears valid to conclude, in view of these considerations, that by the criterion of decreased susceptibility to beta-amylase action bread containing 0.5% emulsifier did not stale at an appreciably lower rate than similar bread not containing this supplementation. This conclusion is in agreement with the recent data of Volz and Ramstad (21) obtained by chemical measurement of reducing substances resulting

from the action of takadiastase and pancreatin on bread crumb. Bread baked by the basic formula with omission of shortening displayed essentially the same rate of decrease in starch susceptibility to beta-amylase during staling as did control loaves. In subjective "squeeze" and "crumb softness" tests, the bread containing emulsifier was consistently rated first, the control containing shortening but no emulsifier second, and the bread containing neither shortening nor emulsifier last. These data suggest that the polyoxyethylene monostearate emulsifiers at the level tested (0.5%) are far more effective in softening the bread than in retarding the starch retrogradation accompanying staling.

Staling of bread resulted not only in a decrease in total amount of starch attackable by beta-amylase (i.e. increase in retrogradation) but also in changes which appeared to make the starch react at a slower rate to saccharifying enzyme action. This is demonstrated in Fig. 4. The data indicate that the percentage of gas produced during the first two hours of a five hour fermentation test decreased with increasing staleness of the bread. In the case of bread not containing emulsifier, 93% of the five-hour gas evolution was produced during the first two hours when the bread was oven-fresh, 72% when the bread was appreciably stale, and 61% when the bread was fully stale. The starch in bread baked with 0.5% polyoxyethylene monostearate displayed the same general decrease in ease of attackability of the starch, but at a somewhat lower level than in the case of bread not containing emulsifier. It may be concluded from these data, in agreement with the data of Fig. 3, that the rates of crumb staling in breads baked with and without addition of emulsifier were not greatly different when evaluated by change in rate of reaction to saccharifying enzyme action.

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RETARDATION OF CRUMB STARCH STALING IN COMMERCIAL BREAD BY BACTERIAL ALPHA-AMYLASE [1, 2]

A. S. Schultz, F. D. Schoonover, R. A. Fisher and S. S. Jackel [3]

ABSTRACT

The addition of small quantities of bacterial alpha-amylase to bread baked on a commercial scale resulted in an improved product which staled at an appreciably decreased rate. Breads baked with the addition of 3 to 6 Sandstedt, Kneen and Blish units of bacterial alpha-amylase per pound of bread were consistently scored fresher, in subjective crumb softness tests and in measurements of starch susceptibility to beta-amylase action by the fermentation test, than breads not containing this supplementation. High levels of bacterial alpha-amylase addition decreased the rate of change in starch susceptibility further than low levels, but resulted in poor quality breads characterized by sticky and gummy crumbs. The applicability of bacterial alpha-amylase supplementation at low levels to the retardation of starch susceptibility changes in a variety of baked goods was established. A preliminary explanation for the favorable behavior of this enzyme at low levels was proposed, based on the high thermostability of bacterial alpha-amylase and on the known susceptibility of gelatinized starch to enzymatic degradation into lower molecular entities.

It is well established that the staling of bread is accompanied by retrogradative changes which decrease the susceptibility of the crumb starch to enzymatic attach (3, 8, 15). Recent data (4) obtained by the yeast fermentation method demonstrated, in agreement with the earlier report of Schultz and Landis (12), that the diminished susceptibility of aged bread crumb to the action of beta-amylase is characterized by a decrease in total attackable carbohydrates and further, by a disproportionately large decrease in fragments rapidly attacked by this enzyme. Other recent data (13), obtained by chemical means, indicated that the crumb starch in aged bread is characterized also by a decreased susceptibility to attack by amylases of mold and animal origin.

It is the purpose of this investigation to determine whether low levels of bacterial alpha-amylase can be successfully employed in commercial bread making to retard retrogradation of starch without giving rise to crumb stickiness and gumminess.

[1] Manuscript received December 21, 1951.
[2] A report based on a portion of the data given herein was presented before the Thirty-Seventh Annual Meeting of the American Association of Cereal Chemists, Dallas, Texas, April, 1952.
[3] The Fleischmann Laboratories, Standard Brands Inc., New York 51, New York.

Materials and Methods

Bread Preparation. White bread was prepared on a commercial scale in 50 lb. flour batches by the sponge dough process. Regular bakery plant equipment, involving high speed mixing, dividing, rounding, intermediate proofing and moulding (automatic make-up), was used and commercial schedules were maintained. The basic formula for control loaves has been given previously (4). Alpha-amylase supplemented loaves were baked by the basic formula with addition in the sponge stage of a purified bacterial [*B. subtilis* type] alpha-amylase enzyme in an amount equivalent to 3 or 6 SKB units[4] of alpha-amylase per pound of bread. Higher levels of supplementation, 30 and 60 SKB units per pound of bread, were carried out for experimental purposes. The time of baking was not reduced from the normal period of 30 minutes.

Bread Evaluation. The baked loaves were sealed in moisture-proof cellophane bags after reaching room temperature and stored at 8°C. and at 30°C. for future testing. Evaluation for degree of freshness, using the criterion of starch susceptibility to beta-amylase, was carried out by the yeast fermentation method described previously (4). Physical characteristics of the breads were determined in a commercial bakery unit by conventional scoring procedures.

TABLE I

EFFECT OF BACTERIAL ALPHA-AMYLASE ON RESULTS IN COMMERCIAL SCALE BAKING OF WHITE BREAD

	Bacterial Alpha-Amylase Supplementation per Pound of Bread			
	0 SKB	3 SKB	6 SKB	60 SKB
Four hour sponge fermentation				
1st hour rise—inches	6.4	6.5	6.5	6.5
2nd hour rise—inches	9.6	9.0	9.4	10.6
3rd hour rise—inches	12.8	12.0	13.0	13.6
Proof time—minutes	72.0	72.0	73.0	73.0
Dough mixing time—minutes	12.5	12.0	13.2	13.2
Handling at make-up machinery	Normal pliability	Normal pliability	Normal pliability	Slightly more pliable
Loaf volume—cc.	2500	2490	2515	2300
Bread pH	5.4	5.4	5.4	5.3
Grain and texture	Normal	Normal	Slightly open	Poor[1]
External appearance	Normal	Normal	Normal	Collapsed
Subjective crumb softness 40 hours after baking	Hardest	Less hard	Soft	Gummy

[1] Fine cell wall but open grain.

[4] Enzyme activity was determined by the Redfern (12) modification of the Sandstedt, Kneen and Blish dextrinizing method.

Results

The data of Table I, showing the effect of bacterial alpha-amylase supplementation at various levels on the quality of the bread, indicate that supplementation in the range of 3 to 6 SKB units per pound of bread retarded the development of crumb hardness normally accom-

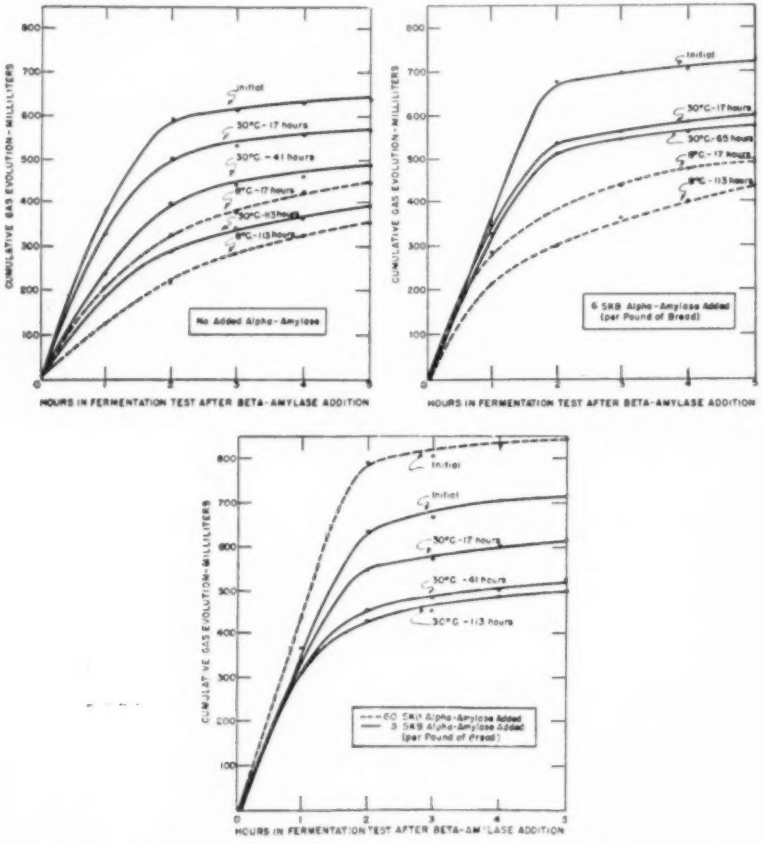
CUMULATIVE GAS EVOLUTION - MILLILITERS
HOURS IN FERMENTATION TEST AFTER BETA-AMYLASE ADDITION
Initial
30°C - 17 hours
30°C - 41 hours
30°C - 113 hours
8°C - 17 hours
8°C - 113 hours
No Added Alpha-Amylase
6 SKB Alpha-Amylase Added (per Pound of Bread)
3 SKB Alpha-Amylase Added (per Pound of Bread)

FIG. 1. Effect of bacterial alpha-amylase on rate of retrogradation of starch in bread crumb.

panying aging without otherwise affecting normal bread characteristics. At these levels there were no apparent changes in sponge fermentation rate, proof time, or dough handling characteristics. There was no difference in behavior or effectiveness when the enzyme was added to the dough stage instead of the sponge stage. Supplementation at ten-fold higher levels, however, resulted in poor grain, poor texture and

sticky and gummy crumbs. It may be concluded from these data that commercial supplementation of white bread with low levels of bacterial alpha-amylase is entirely feasible and may be expected to have no effect on normal bread making operations.

The effect of supplementation with various concentrations of bacterial alpha-amylase on the changes in starch susceptibility to beta-amylase accompanying aging of bread is shown in Fig. 1. It is evident from the curves obtained with bread containing no added alpha-amylase that staling resulted in lessened crumb starch susceptibility to beta-amylase attack, as evidenced by decreased gas production in the fermentation test. Comparison of these curves with those obtained for bread containing 6 SKB units of bacterial alpha-amylase per pound

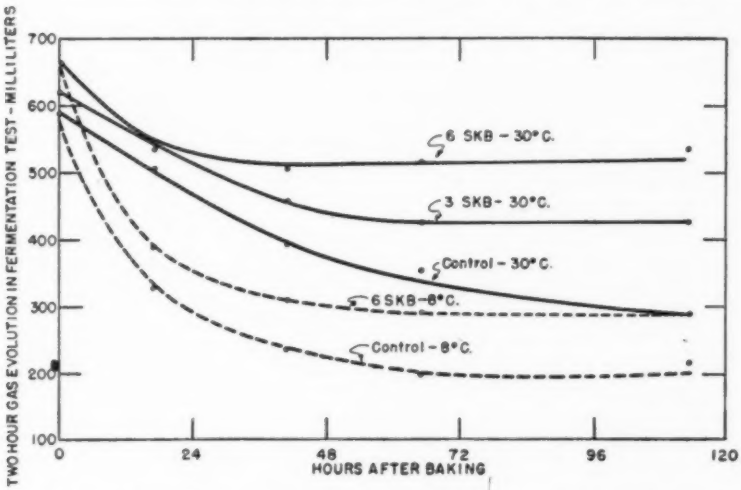

FIG. 2. Effect of bacterial alpha-amylase in retarding retrogradation of crumb starch on storage.

of bread indicates certain striking differences. Gas production, indicative of the state of crumb starch freshness, was higher at each time-temperature period for the supplemented bread than for the control. At the end of the first 17 hours of storage at 30°C. both the supplemented and non-supplemented breads were at reasonably similar states of freshness. Although the susceptibility of the starch in the non-supplemented bread continued to decrease rapidly, the starch in the 6 SKB bread showed little change. The 3 SKB bread was intermediate between the control and the 6 SKB bread in its behavior.

These comparisons are shown somewhat more clearly in the data of Fig. 2. It is evident that at any time interval the bacterial alpha-amylase loaves were fresher than corresponding non-supplemented

breads by the criterion of starch susceptibility to beta-amylase. It is also evident that the amylase supplementation succeeded in maintaining starch susceptibility at a level determined by the amount of supplementation employed. The effect of both of these factors in maintaining crumb starch freshness of the bread is shown in Table II. It may be concluded from these data that the crumb starch in a loaf stored at 30°C. and containing 6 SKB units of bacterial alpha-amylase per pound of bread maintained the same freshness during prolonged storage, as evaluated by starch susceptibility measurements, as a 13 hour old control bread. Under these conditions the crumb starch freshness of a 3 SKB bread was maintained at the same level as a 33 hour old control bread.

TABLE II

EFFECT OF BACTERIAL ALPHA-AMYLASE IN RETARDING RETROGRADATION OF BREAD CRUMB STARCH [1]

Storage Age[2]	Level of Supplementation per Pound of Bread				
	Control-0 SKB	3 SKB		6 SKB	
	Gas Production[3]	Gas Production[3]	Apparent Age[4]	Gas Production[3]	Apparent Age[4]
hrs.	*ml.*	*ml.*	*hrs.*	*ml.*	*hrs.*
0	593	634	—	673	—
12	525	567	4.5	573	3
24	465	516	13	525	12
36	413	472	23	517	13
48	373	442	30	515	13
60	345	428	33	515	13
84	310	425	33	515	13
108	292	425	33	515	13

[1] Data derived from experimental data plotted in Fig. 2.
[2] Storage at 30°C. in moisture-proof cellophane starting about one hour after removal from oven.
[3] Volume of gas evolved in 2 hours after addition of beta-amylase in yeast fermentation method.
[4] Apparent age is defined as the storage age of the control loaf at which it produced the indicated volume of gas.

It has been pointed out recently that staling of bread results not only in a decrease in total amount of starch attackable by beta-amylase but also in changes which appear to make the starch react at a slower rate to beta-amylase action (4). This is clearly demonstrated in the control bread curves of Fig. 3. The percentage of gas produced during the first two hours of a five-hour fermentation test decreased from a value of 93% when the bread was fresh to a value of 74% when the bread had been aged for 113 hours at 30°C. In contrast, this value decreased from 93% to only 85.5% when the bread contained 3 SKB units of bacterial alpha-amylase per pound, and showed essentially no decrease when excessively large alpha-amylase concentrations were used. From these data, and from those of Table III, it is apparent

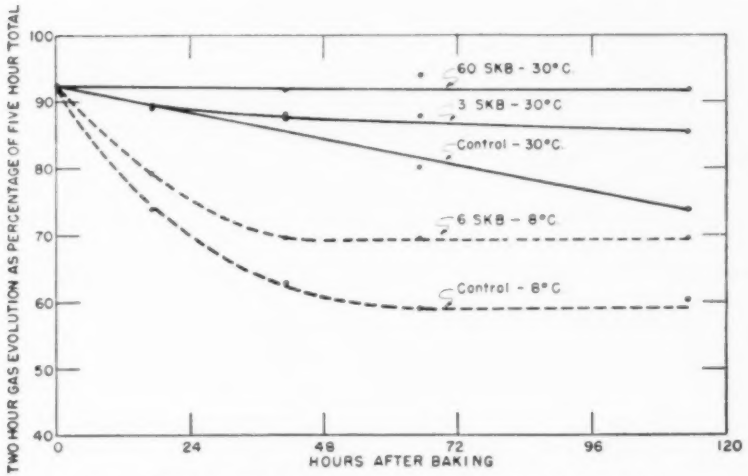

FIG. 3. Effect of bacterial alpha-amylase in maintaining ease of attackability of crumb starch by beta-amylase.

that the alpha-amylase supplemented loaves remained appreciably fresher on storage, with respect to changes in rate of reaction to beta-amylase action, than did the nonsupplemented controls. By this criterion the crumb starch freshness of a 6 SKB loaf was maintained over prolonged storage at the 17 hour control bread level.

TABLE III

EFFECT OF BACTERIAL ALPHA-AMYLASE IN MAINTAINING EASE OF ATTACKABILITY OF BREAD CRUMB STARCH [1]

Storage Age[2]	Level of Supplementation per Pound of Bread						
	Control-0 SKB	3 SKB		6 SKB		60 SKB	
	2:5 Hour Ratio[3]	2:5 Hour Ratio[3]	Apparent Age[4]	2:5 Hour Ratio[3]	Apparent Age[4]	2:5 Hour Ratio[3]	Apparent Age[4]
hrs.	%	%	*hrs.*	%	*hrs.*	%	*hrs.*
0	92.6	92.6	0	92.7	0	92.9	0
12	90.4	90.4	12	90.4	12	92.9	0
24	88.4	88.6	22	89.7	16	92.5	0
36	86.4	87.6	28	89.6	16	92.3	1
48	84.5	87.3	30	89.5	17	91.8	2
60	82.5	86.9	33	89.5	17	91.7	2
84	78.6	86.3	37	89.5	17	91.7	2
108	74.7	85.6	42	89.5	17	91.7	2

[1] Data derived in part from experimental data plotted in Fig. 3.
[2] Storage at 30°C. in moisture-proof cellophane starting about one hour after removal from oven.
[3] The percentage ratio of the volume of gas evolved in 2 hours after addition of beta-amylase to that evolved in a 5 hour total in the yeast fermentation method.
[4] Apparent age is defined as the storage age of the control loaf at which the same 2:5 hour ratio was obtained.

In view of the known thermal stability (14) of bacterial alpha-amylase it appeared advisable to collect experimental evidence bearing on the question of retention of amylase activity in supplemented bread after baking. Johnson and Miller (6) reported that 39% of the alpha-amylase activity in a special flour extract supplemented with high levels of bacterial alpha-amylase (estimated at equivalent to more than 30 SKB units per pound of bread) resisted inactivation when the temperature was gradually raised to 176°F. (80°C.) in an amylograph. These data were confirmed by measurements, using the liquefying method (10), on bread supplemented with 60 SKB units per pound of bread. Retention of alpha-amylase activity at this excessively high level was approximately 45%. Similar measurements[5] on bread supplemented at the commercially recommended level, 6 SKB units per

TABLE IV

EFFECT OF ADDITIONAL BAKING AND TREATMENT IN FLOWING STEAM ON EVOLUTION OF GAS IN FERMENTATION TEST [1]

Experimental Conditions	Volume of Gas Evolved in 2 Hours	
	Control Bread	6 SKB Bread [2]
	ml.	*ml.*
Normal bake—30 min.	345	510
Over bake—40 min.	333	495
Normal bake	343	488
Normal bake + 30 min. treatment in flowing steam	328	485

[1] Breads tested after 65 hours storage at 30°C.
[2] Bread supplemented with 6 SKB units of bacterial alpha-amylase per pound of bread.

pound of bread, indicated that small amounts of enzyme, representing a retention of approximately 20%, were present after baking.

Since alpha-amylase is known to function synergistically with beta-amylase it appeared necessary to determine whether the traces[6] of activity remaining after baking in bread supplemented with 3 to 6 SKB units of bacterial alpha-amylase were sufficient to have an appreciable influence on the results in the yeast fermentation method used to evaluate changes in bread crumb starch susceptibility to beta-amylase. Preliminary evidence indicating that residual alpha-amylase was not a source of error in the fermentation method was obtained by experiments in which bread was baked for an additional ten minutes and by experiments in which oven-fresh bread, sealed in moisture-proof

[5] We are indebted to Dr. Sutton Redfern for making the residual alpha-amylase determinations.
[6] Based on a retention of 20% it is estimated that the analytical sample (20 g.) of crumb from bread baked with 3 to 6 SKB units of bacterial alpha-amylase per pound would contain not more than 0.05 SKB units of enzyme activity.

cellophane, was exposed to flowing steam for 30 minutes. These experimental conditions might be expected to result in further thermal inactivation of the enzymes and thereby reduce the amount of residual alpha-amylase. The data in Table IV, indicate that gas evolution in the fermentation test was not reduced by either of these treatments and may be interpreted as evidence against residual alpha-amylase influence on the results of the test.

The data of Table V present positive evidence that traces of residual alpha-amylase have little or no effect in the fermentation test on the

TABLE V

EFFECT OF ADDITION OF BACTERIAL ALPHA-AMYLASE DURING TEST ON GAS EVOLUTION IN FERMENTATION TEST [1]

Alpha-Amylase Addition		Test Conditions		Volume of Gas Evolved [2]		
Equivalent Activity per Lb. of Bread	Simulated Retention After Baking	pH	Storage at 30°C	Control Bread	6 SKB Bread [3]	60 SKB Bread [3]
SKB units	%		*hrs.*	*ml.*	*ml.*	*ml.*
0	0	5.2	23	459	520	—
3	50	5.2	23	532	539	—
6	100	5.2	23	562	541	—
0	0	5.2	77	347	524	—
3	50	5.2	77	376	523	—
6	100	5.2	77	399	515	—
0	0	4.0	47	385	479	—
3	50	4.0	47	378	489	—
6	100	4.0	47	402	502	—
0	0	5.2	95	281	506	788
0	0	4.0	95	273	478	756

[1] Normal pH in the fermentation test is 5.2. Data reported are two hour gas evolutions except as noted.

[2] Three hour gas evolution data are given for measurements at pH 4 because of slower rate of fermentation at this pH as compared to pH 5.2.

[3] Breads supplemented with 6 or 60 SKB units of bacterial alpha-amylase per pound of bread as indicated.

behavior of bacterial alpha-amylase supplemented breads. Addition during the test, of an amount of bacterial alpha-amylase equal to 6 SKB units per pound of bread and representing the equivalent of 100% retention of enzyme activity after baking, increased the gas evolution from non-supplemented breads but had no significant effect on the gas evolution from supplemented bread. This observation, in view of other data[7] showing that increased gas production resulted from both supplemented and non-supplemented bread when twenty-fold larger amounts of alpha-amylase were added during the test, indi-

[7] Jackel, S. S., Schultz, A. S., and Schaeder, W. E., in preparation.

cates that the nature of the crumb starch in supplemented bread is altered sufficiently so that the presence of low levels of alpha-amylase during the test is without effect.

Examination of the data for the control bread indicates that addition during the test of an amount of alpha-amylase equivalent to 6 SKB units per pound of bread increased gas production by 103 ml. (from 459 to 562 ml.) when the bread was 23 hours old but only by 52 ml. (from 347 to 399 ml.) when the bread was 77 hours old and relatively stale. These data suggest that normal aging of bread is accom-

TABLE VI

EFFECT OF BACTERIAL ALPHA-AMYLASE IN RETARDING CRUMB STARCH STALING OF VARIOUS TYPES OF BREAD

Type	Bacterial Alpha-Amylase Supplementation	Storage at 30°C	Gas Evolution[1]	Ratio of 2:5 Hour Gas Evolution[2]	Subjective Freshness Preference	
					Squeeze	Crumb[3]
	SKB units/ lb. of bread	*hrs.*	*ml.*	*%*		
Rye	0	89	175	54.8	2	2
	3	89	463	82.9	1	1
Vienna	0	41	278	66.6	3	3
	3	41	390	78.3	2	2
	6	41	475	80.2	1	1
White	0	41	325	63.8	4	4
	0[4]	41	385	72.4	1	2
	3	41	442	78.2	3	3
	6	41	486	83.7	2	1
Whole wheat	0	76	291	73.1	3	3
	3	76	327	79.2	2	2
	6	76	402	86.1	1	1

[1] Volume of gas evolved in 2 hours after addition of beta-amylase in yeast fermentation method.
[2] The percentage ratio of the volume of gas evolved in 2 hours after addition of beta-amylase to that evolved in a 5 hour total in the yeast fermentation method.
[3] Evaluated by subjective feel of the crumb between the fingers and by subjective feel and visual observation of the crumb springback. Fresh bread crumb is characterized by a silky or velvety feel and by low springback, changing as the bread ages, to a harsh and crumbly feel and increased springback.
[4] This bread contained 0.5% polyoxyethylene monostearate emulsifier.

panied not only by a decrease in susceptibility of the crumb starch to attack by beta-amylase but also by a disproportionately large decrease in susceptibility to attack by joint action of both beta- and alpha-amylase. These observations provide further evidence against traces of residual alpha-amylase having a significant influence in the fermentation test, since the effect of the enzyme during the test would become progressively less as the bread staled whereas in fact, as shown in Fig. 2, the difference in gas production between supplemented and non-supplemented loaves increased with increasing age. Further data,[7]

obtained with twenty-fold larger amounts of alpha-amylase added during the test, are in agreement with the data reported and substantiate the conclusions suggested.

The results obtained at pH 4.0, acidity conditions which are unfavorable to bacterial alpha-amylase activity, indicate that enzyme added during the test at this pH was without influence on the gas evolution from supplemented or non-supplemented bread. It may be concluded, therefore, from the final data of Table V in which identically large differences between supplemented and non-supplemented loaves were obtained at the normal pH, 5.2, and at pH 4.0, that traces of residual alpha-amylase do not influence the results of the fermentation test.

Table VI summarizes data obtained in connection with a variety of different types of breads baked on a commercial scale by conventional formulations. It is evident that bacterial alpha-amylase supplementation was effective in each case in maintaining crumb starch susceptibility to beta-amylase digestion during aging of the bread.

Discussion

The practice of supplementing flour or dough with natural materials having alpha-amylase activity, such as germinated cereals and malt syrups, has achieved widespread commercial acceptance because of the resulting improved loaf characteristics (9). Johnson and Miller (6) have recently advocated the commercial use of alpha-amylase from fungal sources for this purpose, pointing out certain advantages over supplementation with malted cereals. Supplementation with bacterial alpha-amylase at the high levels used for diastating or viscosity purposes in connection with loaf quality improvement has been unsuccessful (2, 5). The greater thermostability of the bacterial alpha-amylase, as compared to the malt enzyme, resulted in excessive starch breakdown during baking and consequent unsatisfactory grain and texture in the finished bread. Kneen and Sandstedt (9), however, have pointed out that the action of bacterial amylases appears to be quite comparable with those of other alpha-amylases during dough fermentation.

The levels of bacterial alpha-amylase recommended in the present paper for use in connection with the prevention or retardation of crumb starch staling during aging of bread, are approximately ten-fold less than those described previously. At these low concentrations, as indicated in the data of Table I, crumb gumminess and stickiness did not develop and the breads were completely satisfactory and normal.[8] It would appear from these results that commercial supplementation of

[8] Conn, Johnson and Miller (2) have reported obtaining slightly sticky crumbs when supplementation with thermolabile bacterial alpha-amylases, designated Diastase-28 and -30, was carried out at similar low levels. It would appear, since we have never encountered sticky crumbs with our thermostabile bacterial alpha-amylase enzyme at these levels, that the bacterial species origin or degree of purity of the enzyme may be involved.

bread with low levels of bacterial alpha-amylase is feasible and offers, for the first time, a practical method for effective control of the starch changes occurring during the aging of bread. The significance of this finding is self-evident in view of Katz' report (7) that retrogradation of starch is the prominent factor bringing about staling and in view of Bradley's definition (1) of a true anti-staling agent as one which decreases the rate of retrogradation.

The nature of the mechanism by which low levels of bacterial alpha-amylase function to retard crumb starch changes during aging of bread is not altogether clear and is being studied further. It appears probable, since the thermolabile alpha-amylase enzymes do not produce an anti-staling action even at significantly higher levels, that the favorable action of bacterial alpha-amylase must be associated with its high thermostability. The most reasonable explanation at present is that the alpha-amylase action does not take place during baking until a sufficiently high temperature has been reached to gelatinize the starch and make it accessible to attack. Since this temperature is sufficiently high to inactivate non-bacterial but not bacterial alpha-amylase, it is likely that the bacterial enzyme is the principal amylase present in an active form for any appreciable length of time at the temperature at which the starch is susceptible to alpha-amylase action.

It is known that the action of alpha-amylase on starch is hydrolytic in nature resulting in the formation of a variety of degradation products, ranging in size from molecules of high molecular weight to dextrins containing approximately six glucose residues. It appears reasonable to postulate that the bacterial alpha-amylase functions during baking by modifying the gelatinized starch molecules sufficiently to result in an increased proportion of low molecular weight fragments characterized by decreased retrogradation rates. Forthcoming data,[7] indicating that the crumbs of supplemented bread contain appreciably greater amounts of reducing substances than do untreated bread crumbs, tends to support this hypothesis. It is also possible that the residual bacterial alpha-amylase in the finished bread functions during storage of the bread to augment the effect on starch susceptibility produced initially by the enzyme during baking.

In conclusion, it appears that by appropriate choice of the amount of bacterial alpha-amylase supplementation it is possible to maintain the starch freshness at the oven value or the day old or two day old value or at any desired intermediate level. The versatility of the product in this respect permits the production of bread that will satisfy regional consumer preference and affords the baker a tool for controlling the starch freshness of his product.

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GLUTEN FRACTIONS OF HARD RED SPRING AND DURUM WHEAT AND THEIR BAKING QUALITY[1, 2]

R. H. HARRIS and DONALD FROKJER[3]

ABSTRACT

Hard red spring wheat gluten dispersed more readily in lactic acid solutions than durum wheat, and the solubility extended over a greater range in pH. Lactic acid 0.013 *N* was more satisfactory than acetic or formic.

Hard red spring wheat gluten improved the baking quality of soft wheat flour more than durum gluten did. Three fractions were obtained from lactic acid dispersion by raising the pH level in three successive stages, with calcium hydroxide. These pH levels were 5.0, 5.0 to 5.5, and over 5.5. For both wheat glutens, fraction II was markedly superior to the other fractions, comparing favorably with the original unfractionated gluten, while fraction I was very inferior. Purifying the gluten by precipitation with calcium hydroxide from dispersion did not improve the baking quality.

Durum gluten was slightly higher in proteolytic activity than hard red spring gluten. There were no differences among the durum fractions in activity. For the hard red spring, fraction I was highest, while fraction III showed the lowest proteolytic activity of any samples. In reducing groups, fraction I was highest, while the durum gluten as a whole had more reducing groups than the hard red spring gluten. The differences in proteolytic activity and reducing action of these fractions were unrelated to their effects on baking quality. Information obtained from samples of fraction I separated from gluten dispersed on a rotary shaker without foaming, from redispersion at pH 7.0, and from a dispersion heated for ten minutes at 95°C. to destroy proteolytic activity, did not reveal the cause of the inferior baking quality of fraction I.

Lusena (11) has recently described a method for preparing wheat gluten for baking purposes based upon the separation of gluten dispersed in acetic acid by adjusting the pH to the iso-electric point with calcium hydroxide. It appeared probable to the authors that by gradually raising the pH by suitable additions of this reagent to dispersed gluten, fractions of different solubilities and characteristics could be obtained.

The present paper deals with the application of a modified form of Lusena's method of separating and purifying gluten for the preparation of gluten fractions from hard red spring and durum wheat, and the

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[2] Contribution from the North Dakota Agricultural Experiment Station, and School of Chemical Technology, North Dakota Agricultural College, Fargo, North Dakota.

[3] Cereal Technologist, and graduate student respectively.

determination of their baking quality when blended with a low protein soft wheat flour.[4]

Materials and Methods

Materials. The glutens were prepared from two composite flours. The hard red spring composite had been experimentally milled from samples of Mida, Thatcher, Rival, Cadet, Pilot and Redman grown at various locations in North Dakota in 1949. The durum composite consisted of durum flour and semolina reduced to flour fineness, and included Mindum, Carleton, Stewart, Nugget and L.D. 308, grown at three stations also in 1949. The protein contents of the three flours were as follows expressed on 14.0% moisture basis.

Hard red spring	12.8%
Durum	13.4%
Soft wheat (Golden)	7.2%

Reagents employed were of A.R. purity; none of them were redistilled or otherwise repurified.

Methods. The glutens were washed from doughs which had stood under a sodium phosphate solution of pH 6.8 for one hour at 30°C., using the method of Dill and Alsberg (4). After standing for approximately one hour in this solution the gluten was dispersed in the selected acid in a Waring Blendor at low speed for five minutes. Acid at lower pH than for hard red spring gluten was required to completely disperse durum gluten. Five drops of capryl alcohol were added to the dispersion to reduce foam. The dispersion was centrifuged at 3900 r.p.m., and the gluten recovered from the supernatant by addition of saturated calcium hydroxide solution with stirring until the desired pH value was attained (Lusena, 11). After recovery, the gluten was divided into small pieces, placed on waxed paper and dried with fanning at room temperature for 24 hours. The dried pieces were passed once through a Labconco grinder with plates set as for grinding wheat. The dry glutens were analyzed for moisture, and nitrogen content.[5]

The micro malt-phosphate-bromate method with 5% sucrose was employed for ascertaining the baking quality of the glutens. (5) Sufficient hydrated gluten was added to the soft wheat flour to bring the protein level to 13.0% (13.5% moisture basis). The same procedure and absorption were used for mixograms except that distilled water only was employed. Aitken and Geddes (1) showed the utility of adding dried gluten to low protein flour to evaluate the baking quality of the gluten. Because of the restricted quantities of material the micro-baking formula was used. Hydration of the gluten was started

[4] Samples of wheat furnished by E. J. Seeborg, Soft Wheat Laboratory, Pullman, Washington.
[5] Cereal Laboratory Methods, 5th ed., Amer. Assoc. of Cereal Chemists. University Farm, St. Paul 1, Minn.

one hour before mixing (Harris and Sibbitt, 5); the gluten was incorporated in thin strips with the base flour.

Several methods were examined before one was found suitable for preparing the gluten previous to determining the proteolytic activity by measurement of nonprotein nitrogen of the dried gluten. For all methods sufficient gluten to yield a 1.0% concentration of protein was hydrated in 5 ml. of water for 30 minutes before dispersion in 0.105*N* acetic acid. The samples were incubated at 40°C. for 24 hours before

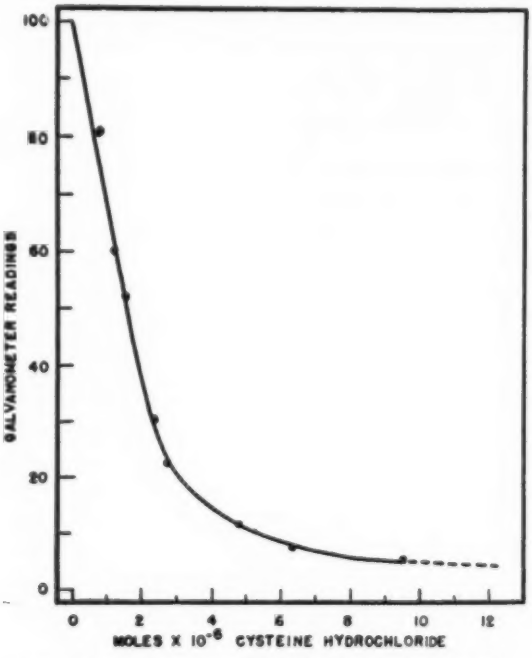

FIG. 1. Calibration curve used for estimation of reducing values.

determining the nonprotein nitrogen by precipitating the protein nitrogen with trichloracetic acid and ascertaining the nitrogen content of the filtrate. For method No. 1 acetic acid dispersions only were employed. The initial pH was 3.12 and the final 3.45. For No. 2, 0.625 g. of bacto-hemoglobin was dispersed with the gluten in the acid. Capryl alcohol was used to depress foaming. Here the initial pH was 3.30 and the final 3.68. Method No. 3 was the modified Ayre-Anderson procedure (12). Method No. 4 resembled No. 2 except that no capryl alcohol was used since statements have appeared in the literature that

this substance interfered with the determination (7). Following is a comparison of the four methods.

Method		*Activity (mg. N/ g. protein)*
1	Acetic acid dispersion, no bacto-hemoglobin	6.2
2	Acetic acid dispersion with bacto-hemoglobin and capryl alcohol	28.6
3	Modified Ayre-Anderson procedure	2.0
4	Same as method 2 except no capryl alcohol	28.5

It is probable that the presence of buffer salts depressed gluten solubility and interfered with the determination in Method 3. Method 2 was the most satisfactory, because it greatly reduced the time required for the foam to subside, and it was employed for assessing the proteolytic activity of the dried gluten samples.

The reducing groups were ascertained by the colorimetric method proposed by Chapman and McFarlane (2) and modified for flour and gluten by Hlynka and Bass (9). Fig. 1 shows the calibration curve obtained with cysteine hydrochloride. The aliquot of gluten dispersion was adjusted so that the reducing groups as cysteine hydrochloride fell on the linear portion.

Results

Comparative Dispersability. The method described by Lusena (11), was satisfactory for hard red spring wheat gluten but it was impossible

TABLE I

COMPARATIVE DISPERSABILITY OF DURUM WHEAT GLUTEN IN ORGANIC ACIDS

Concentration g./50 ml.	pH	mg. N / ml.	Gluten Dispersed, %	pH	mg. N / ml.	Gluten Dispersed, %	pH	mg. N / ml.	Gluten Dispersed, %
Normality 0.0125									
	Acetic			Lactic			Formic		
4.5	4.3	2.3	57.9	3.3	3.6	88.4	4.2	2.1	49.0
6.5	4.5	2.6	44.7	3.5	4.7	81.2	4.9	1.4	22.3
8.5	—	—	—	3.8	5.7	75.5	—	—	—
Normality 0.025									
4.5	3.8	2.8	68.3	3.0	4.0	96.1	3.8	4.0	93.8
6.5	3.9	4.7	82.4	3.2	4.6	78.8	3.9	4.2	67.2
8.5	4.0	3.9	52.5	3.8	5.7	76.1	4.3	2.8	34.6
Normality 0.050									
4.5	3.4	4.0	100.0	2.8	3.6	87.4	2.8	3.5	82.2
6.5	3.6	4.8	83.4	2.8	4.6	79.1	2.8	4.8	77.9
8.5	3.6	6.3	85.9	2.9	5.7	76.1	2.9	5.9	73.4

to disperse durum gluten quantitatively in either of 0.005*N* or 0.010*N* acetic acid used by Lusena. Since Cunningham and Anderson (3) have reported that formic acid was useful for dispersing rye and barley gluten, it was decided to include formic and lactic with acetic to ascertain the best acid and pH to employ with durum gluten. The results from using the three acids at three normalities are shown in Table I. As suspected, dispersion was influenced by both pH and the anion of the acid. Complete dispersion was not attained above pH 3.8 for any acid. For acetic and formic acid the amount of gluten dispersed varied

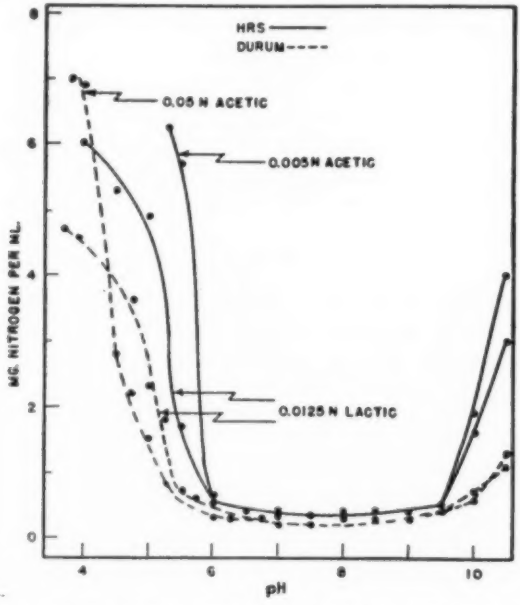

FIG. 2. Solubility of hard red spring and durum gluten in acetic and lactic acid following addition of $Ca(OH)_2$ solution to various pH levels. Normalities are of original acid.

directly with normality, but for lactic acid, normality had a smaller influence on the dispersion. The foam in the lactic acid dispersions also subsided more rapidly. It was observed that the durum gluten separated most easily from lactic acid. The solubility of the two types of wheat gluten in lactic and acetic acid at different pH levels obtained by adjusting the pH of the dispersions with calcium hydroxide is shown in Fig. 2. Apparently the durum gluten was dehydrated more readily than the hard red spring gluten. The minimum solubility range for the durum gluten was greater than for the hard red spring. For the preparation of gluten fractions for baking quality determinations,

0.013*N* lactic acid was employed as the dispersion medium. This was chosen, instead of a higher concentration, because use of large quantities of calcium hydroxide required to neutralize these stronger acids was undesirable.

Gluten Fractionation. By separation of fractions by adjusting pH levels, contamination with inorganic salts is largely avoided. Three fractions were obtained by selecting arbirtary pH levels in lactic acid dispersions since both types of wheat gluten exhibited similar dispersion curves in this acid (Fig. 2). Unfractionated glutens were prepared by direct drying and by purification by Lusena's method. The protein contents, with the percentages of the different fractions obtained by adjustment of the pH levels were as follows:

	Gluten Distribution		*Protein Content (Dry Basis)*	
	Hard Red Spring %	*Durum* %	*Hard Red Spring* %	*Durum* %
Crude gluten			78.6	74.3
Purified gluten			88.8	83.9
Fraction I (pH 5.0)	6.0	10.0	73.5	79.6
Fraction II (pH 5.0 to 5.5)	81.0	54.0	88.0	76.3
Fraction III (pH 5.5 to 7.0)	13.0	36.0	96.2	94.3

It was observed that the protein in fraction I for both wheats was more hydrated and less tenacious and elastic than the other fractions, while fraction III was markedly lacking in elasticity.

Baking Quality. The results from baking the blends fortified with dried gluten to a protein level of 13.0% are shown in Table II while the loaves are represented in Fig. 3. Bakings were in triplicate except

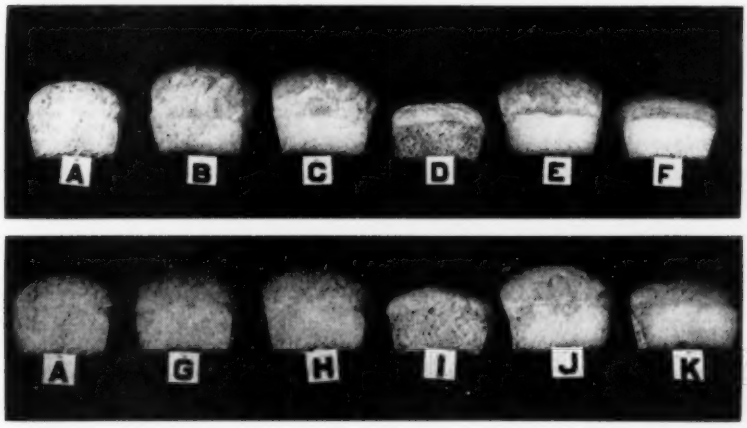

FIG. 3. Loaves from the soft wheat flour and wheat gluten blends. A—control—no gluten added; B and G—with crude gluten; C and H—with purified gluten, D and I—with fraction I, E and J—with fraction II, F and K—with fraction III. Upper row: hard red spring. Lower row: durum.

for fraction I, which was restricted in quantity. The absorptions were approximately 3% lower for the durum gluten blends than for the hard red spring wheat. Fraction III of the hard red spring wheat had lower absorption than the other two fractions for both wheats. The crude and purified gluten blends produced loaves of equal volume which were much larger than the control. The hard red spring wheat gluten blend was significantly stronger than the durum gluten blend as might be expected from previous knowledge of the baking quality of durum wheat flour

TABLE II

RESULTS OBTAINED FROM BAKING THE GLUTEN AND SOFT WHEAT FLOUR BLENDS
(All Loaves Were Overoxidized)

Gluten Added	Absorption	Dough Characteristics	Loaf Volume	Grain and Texture[1]	Crumb Color[2]	General Appearance	
						Crust Color[3]	Symmetry
	%		cc.				
Control (no gluten)	55.2	Weak	130	5.0	7.0	P	2.0
Hard Red Spring Wheat Gluten							
Crude	61.6	Very good	173	7.0	7.0	S	4.5
Purified	61.6	Very good	173	7.0	8.0	S	4.5
Fraction I [4]	61.6	Poor	98	2.5	3.5 g	P, D	1.0
Fraction II [4]	61.6	Excellent	175	8.0	8.0	S	4.5
Fraction III [4]	56.8	Poor, slack	130	4.0 C, O	6.0 g, y	P, G	1.5
Durum Wheat Gluten							
Crude	58.8	Good	153	6.0 C, O	4.5 g	D	3.5
Purified	58.8	Fair	150	7.0	7.0	D	3.5
Fraction I [4]	58.8	Poor	110	2.5	3.5 g	P, D	1.0
Fraction II [4]	58.8	Good	147	6.0 C, O	6.0 g, y	D	2.5
Fraction III [4]	58.8	Dead	128	4.0 C, O	6.0 g, y	D, G	1.5

[1] Grain & texture: O—open, C—closed.....perfect score 10.
[2] Crumb color: y—yellow, g—grey.....perfect score 10.
[3] Crust color: S—satisfactory, P—pale, D—dull, G—glossy.
[4] Fraction I was obtained at pH 5.0; fraction II at pH 5.0 to 5.5; fraction III at pH 5.5 to 7.0.

(6). Fraction I from hard red spring wheat gave an extremely poor loaf with low volume, while the corresponding durum fraction was somewhat better, but still inferior to the unblended soft wheat flour. Fraction II produced loaves equal in quality to the original glutens with the volume for the durum gluten blends being lower than for the hard red spring blends. (This difference was caused no doubt by the better baking quality of the hard red spring wheat gluten.) For fraction III the loaves resembled the control, and indicated that little strength was added by this material.

The grain and texture, crust and crumb color, and symmetry scores were generally higher for the hard red spring gluten blends. The purified glutens yielded higher grain and texture and crumb color scores than the crude preparations. Improvement of color doubtless resulted from removal of pigments. The dough handling properties of the fraction I blends, as well as the appearance of the loaves produced from them, suggested that excessive proteolytic activity might be the cause of the inferior quality of these blends.

Mixograms. Mixograms obtained from the blends used in the baking tests are shown in Fig. 4. Surprisingly strong curves were obtained with the hard red spring unfractionated gluten blends. Slight improvement was also evident for the corresponding durum blends. Fraction II from the hard red spring wheat produced a mixogram very similar to the original gluten, while the blends of other fractions had markedly inferior mixing properties, and yielded curves inferior to those

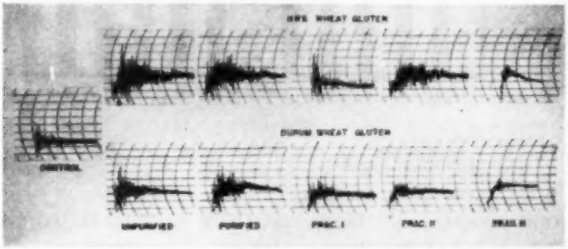

FIG. 4. Mixograms of soft wheat flour and hard red spring and durum gluten blends.

of the original soft wheat flour. Of the durum fractions, the first two had similar mixing characteristics, and the mixograms for the blends were poorer than for the flour alone. Fraction III produced the worst curves for both wheat glutens. Generally, the hard red spring wheat gluten blends had greater dough development periods and higher mixograms than the durum gluten blends. The range of stability was equal for both types of wheat.

Proteolytic Activity. Inquiry into the cause of the marked reduction of loaf volume with fraction I was the next step in this investigation. Olcott, Sapirstein and Blish (13) found that the proteinase appeared to be associated with the glutenin portion of gluten, which would roughly correspond to fraction I. Figure 5 shows the proteolytic activity of the dried glutens. The activity was significantly higher in fraction I than in the other fractions of the hard red spring wheat glutens. Fraction III was much lower in activity than the others, probably because of removal or denaturation of the enzyme as

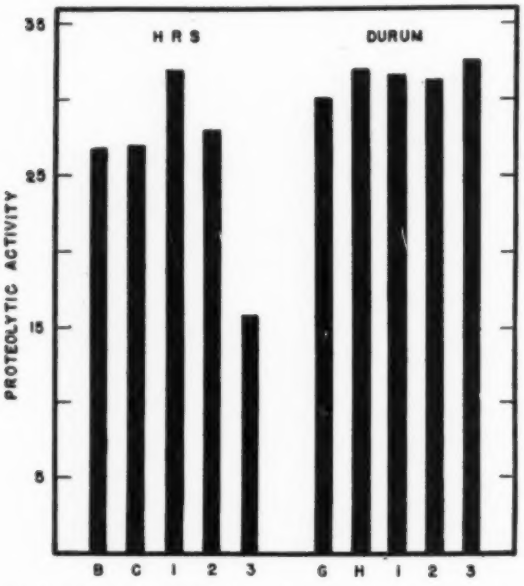

FIG. 5. Proteolytic activity of hard red spring and durum glutens and their respective fractions.
B and G—crude gluten.
C and H—purified gluten.
1, 2, 3, are the respective fractions.

fractionation progressed. However, there were no differences among the three fractions of the durum gluten. The proteolytic activity of the durum gluten in general was slightly higher than for the hard red spring wheat gluten. Apparently gluten proteinase was not chiefly responsible for reducing the loaf volume of blends made with Fraction I.

Reducing Action. Several investigators have shown that the action of reducing groups and proteolytic enzymes are very similar. Hlynka (8) and Sullivan *et al.* (14) found that reducing groups have a very detrimental effect on gluten quality. The data are shown below:

	Reducing Values (Moles × 10^{-6} Cysteine Hydrochloride/g. Protein)	
Gluten Sample	*Hard Red Spring*	*Durum*
Crude	1.90	2.40
Purified	1.25	1.40
Fraction I	2.20	1.95
Fraction II	1.10	1.40
Fraction III	0.45	0.40

Reducing value of the fractions was not correlated with loaf volume of their blends. Fraction I contained more available reducing groups than the other fractions. While this method is non-specific for reduc-

ing groups in gluten proteins, the durum crude gluten had the highest reducing value of any of the preparations but yet improved loaf volume.

Effect of pH. James and Huber (10) reported that acidity increased the fermentation rate of flour-water suspensions, and that the effect varied markedly with the type of flour. Lusena (11) found that hard red spring wheat gluten neutralized to pH 6.8 still contained 0.5% acetic acid, and this increased gluten solubility in distilled water. It might be expected that fraction I would contain more bound acid because it was composed of highly hydrated particles and had been separated at pH 5.0.

The pH of the gluten preparations was determined after hydrating equal weights of each in 10 ml. of water for one hour. The values obtained were as follows:

	Hard Red Spring	*Durum*
Crude	6.1	6.3
Purified	6.1	6.8
Fraction I	4.5	5.0
Fraction II	5.7	5.1
Fraction III	6.7	6.8

Fraction I appeared to merit further study since it was lowest in pH. Experiments with gluten hydrated with lactic acid at different pH levels indicated that the pH of the original gluten had little effect on fermentation of the blends. A portion of fraction I was redispersed

Gluten pH	*Dough at Pan pH*
4.0 (lactic acid)	4.69
5.0 (lactic acid)	4.71
Without acid	4.62

in 0.0125*N* lactic acid and reprecipitated at pH 7.0. The gluten was more tenacious and somewhat similar in physical characteristics to fraction II. The loaf volume was improved by separation at the higher pH, but was still below the control. There was no change in proteolytic activity, but the reducing power had increased. It was thought that possibly the extensive foaming associated with dispersion in the Waring Blendor might have caused some denaturation and exposed sulfhydryl groups. As denatured protein is less soluble it would appear in fraction I. Dispersion in a rotary shaker which eliminated foaming was therefore tried. Baking tests revealed no improvement in the dried gluten prepared in this manner. There was a slight increase in proteolytic activity (39.0) and reducing value. Heating the dispersion to 95°C. for ten minutes to destroy proteolytic activity, (13) before removal of fraction I did not improve the baking quality significantly.

Purifying the glutens by dispersion in lactic acid followed by precipitation with calcium hydroxide did not improve their baking quality, and this tedious procedure does not appear to be justified for this purpose. For fractionating gluten, however, the method is useful and yields fractions differing in appearance and behavior.

The results from this investigation raise several pertinent questions for future study. The underlying causes of the damaging effect on baking quality of fraction I is one of these. Is there a true gluten fraction of extremely poor gluten quality which occurs generally in wheat? Are there significant differences in composition among the gluten fractions? Other problems dealing with the separation of additional fractions and their effect on baking quality, the effect of baking method, and various other factors need to be investigated.

Acknowledgments

The authors wish to acknowledge the important contribution of L. D. Sibbitt to this investigation in evaluating the baking potentialities of the gluten samples. They also wish to thank O. J. Banasik for helpful suggestions for separating the glutens.

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THE DETECTION OF INSECT CONTAMINATES IN CEREALS BY A SPECTROPHOTOMETRIC PROCEDURE [1]

GEORGE C. POTTER and J. A. SHELLENBERGER

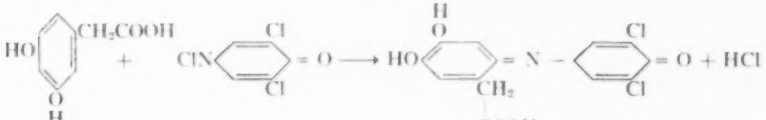
ABSTRACT

A spectrophotometric method of detecting insect material in cereal products is advanced as an alternative to fragment counts. The method depends on the coupling of a substituted quinone with a dihydroxy phenol which occurs in insect cuticle to produce a dye which can be determined spectrophotometrically.

The commonly accepted method for determination of insect fragments in cereals has been the flotation test (5) but the errors inherent in this method are large. A procedure for the detection of insect fragments by means other than recovery of the fragments and actual counting would be of value in laboratories where sanitation analyses are performed. The method presented here is suggested as an alternative to actual counts of fragments.

A number of articles have appeared in the literature postulating the presence of dihydroxy phenols in insect integuments (1, 2, 3, 8, 9, 14). Hackman *et al.* (3) examined a number of insects and found that 3,4-dihydroxyphenylacetic acid was common to all species. Previous to the work of Hackman *et al.*, Schmalfuss and his group (10, 11, 12) had worked on the isolation and characterization of 3,4-dihydroxyphenylacetic acid from the cuticle of various insects.

There is a number of qualitative tests in the literature for detection of phenols. However in general they were not sensitive enough to detect phenols from insects in the concentrations present in cereal products, but the color test of Tillmans and Hirsch (13) proved to be of the required sensitivity. This method consists of forming a phenol-indophenol dye by reaction of the phenol with 2,6-dichloroquinone-chlorimid. The general reaction is:



[1] Manuscript received August 20, 1951. Contribution No. 204, Department of Flour and Feed Milling Industries, Kansas State College, Manhatten, Kansas. This research was supported by a grant from the Millers' National Federation, Chicago, Illinois.

The light absorption peak for the dye formed from 3,4-dihydroxyphenylacetic acid was found to be at 580 mμ.

Materials and Methods

Fifty-gram samples of the cereal to be tested are extracted for 4–6 hours with diethyl ether, using a soxhlet extractor. The ether is removed from the crude fat extract, avoiding overheating of the extract. After removal of the ether, the residue left in the flask is dissolved in 20–25 ml. Skellysolve F. The Skellysolve F is then washed thoroughly with four 25-ml. portions of 1*N* sulphuric acid using a separatory funnel

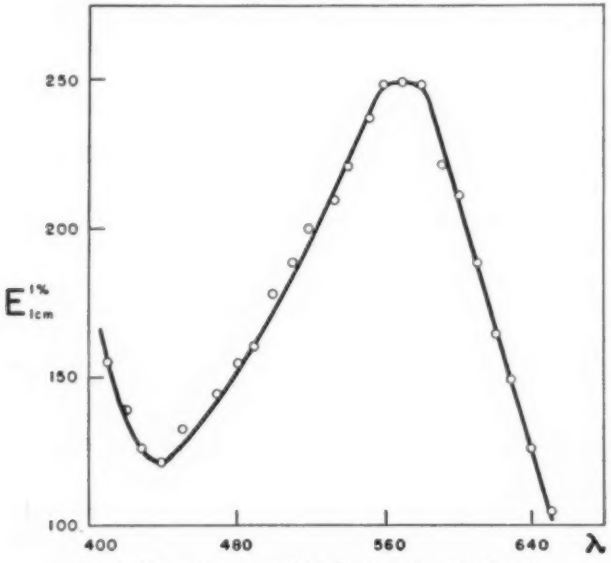


FIG. 1. Absorption spectra of 3,4-dihydroxyphenylacetic acid.

and the Skelly layer discarded after washing with the acid. The 1*N* acid solutions are then extracted three times with Skellysolve F, the Skellysolve being discarded, and then extracted at least three times with diethyl ether using 15–20 ml. portions. Five to 10 ml. of water are added to the ether extract, depending on the final dilution desired. The water must be measured accurately in a pipet for this step. The ether then is removed by heating on a water bath and the remaining aqueous solution filtered into a test tube through a dampened coarse paper.

The water extract is then made alkaline using 5 ml. (pipet) of sodium borate buffer of pH 9.4,[2] 5–6 drops of a solution of 2,6-dichloro-

[2] 19.02 g. sodium tetra borate in 1-l. water or an equivalent amount of borax.

quinonechlorimid [3] are added and the solution filtered through a Whatman No. 5 paper into the cuvettes of a Coleman Model 14 Universal Spectrophotometer and the transmission read at 580 mμ after

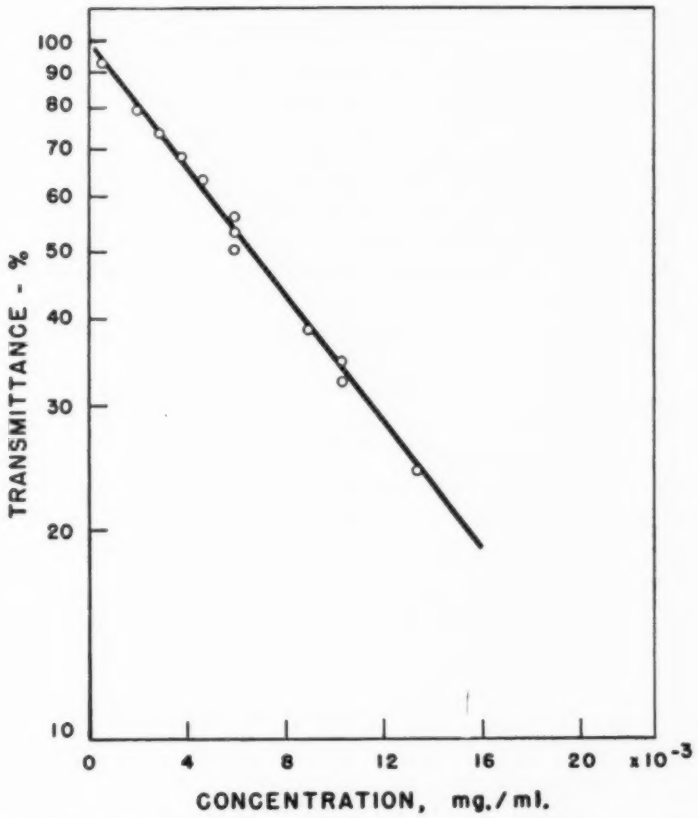


FIG. 2. Standardization curve for 3,4-dihydroxyphenylacetic acid. Coleman Model 14 Universal Spectrophotometer, using 19-mm. cuvettes.

20 minutes. The concentration of the phenol is then determined from the standard curve.

Preparation of Standard.[4] None of the sources contacted had 3,4-dihydroxyphenylacetic acid available.[5] However, 3,4-dimethoxyphen-

[3] 10 ml. distilled water and approximately 0.1 g. 2,6-dichloroquinonechlorimid shaken together and allowed to settle. Use clean portion. Available from Distillation Products Division of Eastman Kodak.

[4] When dissolved in water and left overnight at room temperature molds grow rapidly on the standard; therefore storage in a refrigerator is advisable.

[5] Since this work was completed it has been learned that the Delta Chemical Works, 23 W. 60th St., New York City can supply 3,4-dihydroxyphenylacetic acid.

ylacetic acid can be purchased.[6] The method of Pictet *et al.* (7) was used to demethylate the compound to the phenol.

Two grams of 3,4-dimethoxyphenylacetic acid and 10 g. of hydrogen iodide (sp. gr. 1.5) were refluxed for 1.5 hours. After cooling, saturated sodium acid sulfate solution was added until a yellowish-green color developed. The solution was then extracted with diethyl ether, the ether dried and removed. A heavy oil resulted which slowly crystallized in a desiccator. The material was recrystallized using 1:3 mixture of petroleum ether and benzene. A large amount of solvent is needed to dissolve the material. The resulting material had a M.P. of 124–126°C. while that given in literature was 125–127°C. Figure 1 shows the absorption spectra curve of 3,4-dihydroxyphenylacetic acid. The standard curve resulting from this operation is shown in Fig. 2.

Calculations. Hackman *et al.* (3) found that most common insects contained from 1.4 to 1.9% of a phenol assuming an average figure of 1.6% the amount of insect material can be calculated as follows:

$$\text{Amount of insect material} = 100 \times \frac{\text{amount of phenol}}{1.6}$$

Results and Discussion

In Table I is shown the comparison between the spectrophotometric and fragment count methods for reporting insect matter in

TABLE I

COMPARISON BETWEEN FRAGMENT COUNT AND SPECTROPHOTOMETRIC METHODS FOR DETERMINING INSECT MATERIAL IN CEREALS

Sample	Fragments[1]	Colorimetric
	Frag./50 g.	*mg. insect/50 g.*
1. Bakers patent flour	11	4.00
2. Bakers patent flour	14	3.28
3. Low grade flour	30	6.45
4. A.A.C.C. check sample	7	2.10
5. Cake flour	5	2.25
6. New wheat	2	1.00

[1] Values obtained by the determination described in "Official Methods of Analysis," Association of Official Agricultural Chemists, 7th Ed. (1950).

wheat flour. The values shown are typical of the results by both methods. There is no reason to expect good agreement between the methods in all cases, since the spectrophotometric procedure is based on the presence of phenols in all insect material and not necessarily on the presence of visible insect fragments. A similar situation developed when radiographs (6) of infested wheat kernels were compared with the results of the cracking flotation method (4) using the same wheat.

[6] Distillation Products Div. of Eastman Kodak.

The x-ray technic disclosed much more infestation than the cracking flotation method.

The color formed by coupling of the phenol and substituted quinone is very stable when pure. However, a red color often will develop that can lead to errors. The presence of this color is related to the lipid content of the extract. Filtering such solutions through a Whatman No. 5 filter paper would remove the red color. In several cases it was necessary to repass the filtrate through the paper several times to get a clear blue solution.

Some wheat may contain phenolic compounds that would lead to errors. However a search of the literature failed to produce any quantitative evidence of the existence of such compounds. Catechol was coupled with the quinone and spectrophotometric measurements made. The light transmission of catechol was much less than that of 3,4-dihydroxyphenylacetic acid; therefore, a large amount of catechol would have to be present to cause an appreciable error.

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A SENSITIVE METHOD FOR THE DETERMINATION OF ALPHA-AMYLASE ACTIVITY OF FLOUR[1]

IRWIN STONE

ABSTRACT

A micro dextrinization method based on a procedure suggested by Kneen, Sandstedt and Hollenbeck, suitable for the determination of the traces of alpha-amylase in undiastated and enzyme supplemented flours is described in detail.

Data obtained in tests on flours containing known amounts of added alpha-amylase from both cereal and fungal sources show good recovery of the added enzyme.

Availability of a sensitive method for the determination of the alpha-amylase activity of flour is of importance because of the widespread practice of supplementing flours for baking use with amylases. This paper describes the application for this purpose of the micro dextrinization procedure suggested by Kneen, Sandstedt and Hollenbeck (3).

In the past the main form of supplementation has been the addition of malted cereals to the flour at the mill or in the bakery. There are now being used for this purpose in increasing amounts enzyme preparations derived from fungal sources. Other enzyme sources may also be of interest in the future. The preparations used for supplementation may contain both alpha and beta-amylases, but the amylase which would seem to require more precise control for uniform baking quality is the alpha component.

The amylase activity of flours may be measured by several different types of procedures. The autolytic methods commonly employed involving measurement of evolved gas from a fermenting dough or a determination of "maltose values" following a period of autolytic digestion are measures mainly of the extent of starch damage and the action of the alpha and beta enzyme components in the flour.

For the measurement of alpha-amylase activity of flour, several different methods are available. A viscometric method employing the amylograph has been employed in the evaluation of malt additions to flour by Selman and Sumner (7). Another method which has recently been published by Hollenbeck and Reeder (1) depends on a measure-

[1] Manuscript received October 26, 1951. Contribution from the Wallerstein Laboratories, New York, N. Y.

ment of the sediment volume of an alkali-treated suspension after digestion at 65°C. for one hour. The sediment volume decreases with increasing alpha-amylase content. A related technique, the "dextrin figure" method of Kent-Jones and Amos (2) involves the digestion of an aqueous suspension of the flour at 62°C. for 30 minutes and the subsequent measurement of the amount of dextrin formed. The values obtained were used to indicate whether the flour contained excessive alpha-amylase which would produce a sticky crumb. Flours with dextrin figures below 10 were found satisfactory, between 10 and 14 were borderline cases and those having values over 14 were found to give damp and sticky crumbs.

The values obtained by these various methods have been found to be related to the amount of malt added to the flour, thus giving an index of the alpha-amylase content. While a definite relationship may exist between the measured effect and alpha-amylase activity with a single type of supplementation, this relationship is not valid with enzymes from different sources. In the methods involving the use of high temperatures the results are naturally affected by the heat stability of the particular enzyme system and may not reflect the action at lower temperatures. This is particularly the case for the amylograph and the method is of little value for evaluating fungal amylases for baking use.

The method generally employed for the determination of the alpha-amylase activity of the concentrated enzymes used for flour supplementation is the dextrinization procedure of Sandstedt, Kneen and Blish (6) as modified and brought up to date (5). This method depends on the digestion of a dilute starch solution by a solution of the enzyme in the presence of an excess of beta-amylase and the measurement of the time required for the digest to reach an empirical iodine-color end point.

While this method is satisfactory for malt and other enzymatic products, it is not sufficiently sensitive for the determination of trace amounts of alpha-amylase that exist in flours. Kneen, Sandstedt and Hollenbeck (3) outlined a micro-modification of the dextrinization procedure for the determination of traces of alpha-amylase. This excellent method appears not to have been used to any extent, possibly due to the fact that the procedure was published only in outline and is "buried" in a paper dealing with enzyme stability which an analyst might readily overlook. This micro procedure has been adapted by us as a control procedure for the determination of traces of alpha-amylase in highly purified beta-amylase enzyme. It has been used for a number of years as a means for controlling the purity of Wallerstein Laboratories' Beta Amylase Preparation specified for use in pre-

paring the substrate used in the method for the determination of alpha-amylase (5). This procedure is also extremely useful for the determination of the small amounts of alpha-amylase in flour. It offers the advantage over other methods that the alpha-amylase values are determined by a dextrinization procedure at a low temperature and the results obtained are independent of the heat stability of the particular alpha-amylase or its source. A further advantage is the fact that the same method is used with supplemented flours as in the determination of the activity of the concentrated enzymes which may have been used for the supplementation.

In the macro alpha-amylase dextrinization procedure (5) the enzyme sample reacts with the substrate for a period of time (about 20 minutes) until an iodine-color end point is reached. In the micromethod a preliminary long-time (overnight) digestion of the substrate by the enzyme is employed. This predigested substrate and a blank substrate are then treated with a standard amount of a suitable alpha-amylase preparation of known activity and the end point time is determined. The alpha-amylase activity is then calculated from the difference between the dextrinization time of the predigested substrate and that of a blank containing no added enzyme.

Materials and Methods

REAGENTS

Substrate. Prepare one day prior to using. Place about 400 ml. of water in a 600 ml. beaker and bring to a boil. Add, quantitatively, a cold aqueous suspension of 10 g. (dry basis) of "Merck Soluble Lintner Starch, Special for Diastatic Power Determination." The suspension is added at a rate such that the boiling does not cease. Boil for two minutes. Cool in a water bath while stirring continuously to prevent formation of a skin on the surface. Transfer to a 500 ml. volumetric flask, add 25 ml. of pH 4.6 acetate buffer solution (120 ml. of glacial acetic acid + 164 g. of anhydrous sodium acetate made up to 1-l. with water). Dissolve 0.25 g. of beta-amylase[2] in a small amount of water, add to starch solution and make up to volume. Add a few drops of toluene, shake, and allow to stand at room temperature for 12 to 24 hours before using.

Stock Iodine Solution. Dissolve 11 g. of potassium iodide in about 13 ml. of water. Dissolve 5.5 g. of iodine in this strong iodide solution. When dissolved, dilute to 250 ml. with water. Store in a dark bottle and make fresh solution monthly.

[2] The special beta-amylase enzyme free from alpha-amylase may be obtained from Wallerstein Laboratories, 180 Madison Avenue, New York 16, N. Y.

Iodine Solution B. Dissolve 20 g. of potassium iodide in about 250 ml. of water. Add 2 ml. of the stock iodine solution and dilute to 500 ml. with water.

Extraction and Diluting Solution. Use a 0.2% aqueous solution of calcium chloride.

Standard Enzyme Solution. Prepare immediately before use an enzyme solution containing alpha-amylase from malted cereals, malt diastase, or fungal enzymes[3] by extraction and dilution with 0.2% calcium chloride solution. The activity of this solution is adjusted so that a dextrinization time of 25 to 30 minutes is obtained when 5 ml. of this enzyme solution is added to 30 ml. of the blank determination substrate.

APPARATUS

Reference Color Standard. (4) Use the special Alpha-Amylase Color Disc (catalog 620-S5) made by Hellige Inc., 3718 Northern Blvd., Long Island City, New York, N. Y.

Comparator. Use either the standard Hellige comparator (catalog 607) or the pocket comparator (catalog 605) preferably with prism attachment (catalog 605-A). Illuminate the comparator with a 100-watt frosted lamp mounted in such a manner that direct rays from the lamp do not shine in the operator's eyes. Place the lamp six inches from the rear opal glass of the comparator.

Comparison Tubes. Use precision bore square tubes with a 13 mm. viewing depth.

DETERMINATION OF DEXTRINIZATION END POINT

A portion (1 ml.) of the sample-substrate reaction mixture is added to 5 ml. of dilute iodine solution at 25°C. Mix and transfer to the 13 mm. square tube and compare the color at 25°C. with the alpha-amylase color disc in the comparator. As the end point is approached readings should be taken every half minute. In case two consecutive half-minute samplings show one is darker and the other lighter than the color disc, then record the end point to the nearest quarter minute.

EXTRACTION OF FLOUR

Extract 10 g. of the flour with 100 ml. of 0.2% calcium chloride solution at 20°C. for 15 minutes. Stir the sample every five minutes. At the expiration of 15 minutes, pour the suspension into a centrifuge tube and quickly centrifuge. Filter the supernatant liquor through a

[3] A 0.002% solution of Mylase P may be employed and will approximate the desired dextrinization time. Mylase P is an analytical grade fungal amylase obtainable from Wallerstein Laboratories, 180 Madison Avenue, New York 16, N. Y.

No. 42 Whatman filter paper and discard the first 10 ml. that comes through. If further dilution of the sample is necessary, use the 0.2% solution of calcium chloride.

DETERMINATION

Predigestion. Set up the following in the afternoon noting the time of addition of the flour extract[4] to the substrate. In a series of 125 ml. Erlenmeyer flasks, pipette 20 ml. of the substrate and attemperate at 30°C.[5] Add 10 ml. of sample extract or dilutions thereof at 30°C. and a few drops of toluene. Stopper the flask and hold at 30°C. overnight.

A blank determination is set up by taking 100 ml. of the substrate at 30°C., adding 50 ml. of the 0.2% calcium chloride solution at 30°C. and a few drops of toluene. Stopper the flask and hold at 30°C. overnight.

SECONDARY DIGESTION

Blank. Add 5 ml. of the standard enzyme solution at 30°C. to 30 ml. of the blank substrate which was stored overnight at 30°C. Note the time at the instant of the addition of the enzyme solution. Hold this mixture at 30°C. Determine the dextrinization end point time. Express the blank dextrinization time (t_2) in minutes.

Sample. Add 5 ml. of the standard enzyme solution at 30°C. to the 30 ml. of predigested substrate mixture containing the flour extract which was held overnight at 30°C. and note the time. Hold at 30°C. and determine the dextrinization time (t_3) as above. This dextrinization time will be shorter than the blank determination time by an amount equivalent to the predigestion due to the overnight exposure to the flour enzymes. Record the predigestion time (t_1) which represents the time in minutes from the addition of the sample extract on the previous day to the time of the addition of the enzyme solution for the secondary digestion.

CALCULATIONS

Calculate the alpha-amylase activity by the following formula as units per gram of flour.

$$\text{Alpha-amylase units per gram flour} = \frac{24}{c \times t_1 \times \frac{t_2}{t_2 - t_3}},$$

[4] The weight of sample represented in the 10 ml. aliquot of flour extract should be about 1.0 g. for undiastated flours of low alpha-amylase activity and proportionately smaller charges for more active flours.

[5] We have employed 30°C. because of the convenience of maintaining this temperature for long periods. If a means of maintaining 20°C. is available, this temperature should be employed as this is the temperature recommended in the macro method (5).

where c = grams flour equivalent added to substrate;
t_1 = predigestion time (minutes);
t_2 = blank dextrinization time (minutes); and
t_3 = sample dextrinization time (minutes).

Discussion

Recovery of Added Alpha-Amylase. To test the precision of the micro alpha-amylase method a series of tests were conducted in which several levels of additions of malted wheat and fungal enzymes were made to undiastated flour and the alpha-amylase activities determined. The alpha-amylase activities and the recoveries are listed in Table I.

TABLE I

RECOVERY OF ADDED ALPHA-AMYLASE IN FLOUR

Sample	Alpha-Amylase Activity at 30°C.—Units per Gram					
	Found		Added	Recovery of Added Alpha-Amylase		Average % Recovery
	A	B		A	B	
Wheat Flour A	0.013					
Malted Wheat Flour Preparation (MWFP) 13[1]						
Wheat Flour A plus—						
0.15% MWFP	0.035	0.029	0.010	0.022	0.016	190
0.25% MWFP	0.051	0.045	0.033	0.038	0.032	106
0.40% MWFP	0.076	0.058	0.052	0.063	0.045	104
Fungal Amylase (FA) I = 5250[1]						
Wheat Flour A plus—						
0.005% FA I	0.251		0.262	0.238		91
0.01% FA I	0.560	0.496	0.525	0.547	0.483	98
0.015% FA I	0.696		0.791	0.683		86
0.020% FA I	1.09		1.05	1.08		103
Wheat Flour B	0.007					
Fungal Amylase (FA) II = 1250[1]						
Wheat Flour B plus—						
0.005% FA II	0.076	0.082	0.063	0.069	0.075	114
0.010% FA II	0.133	0.119	0.125	0.126	0.112	95
0.015% FA II	0.190	0.185	0.188	0.183	0.178	96
Wheat Flour C	0.040					
Fungal Amylase (FA) III = 990[1]						
Wheat Flour C plus—						
0.05% FA III	0.534	0.547	0.495	0.494	0.507	*101*
				Grand average = 108%		
				Standard deviation = 15% (of individual results)		

[1] Determined by macro dextrinization procedure.

Except for an occasional result, the recoveries are very good considering the low range of activities being measured. The over-all average recovery amounted to 108% of added enzyme activity with a standard deviation of 15% of individual results.

Acknowledgment

The author wishes to acknowledge the many helpful suggestions of Mr. Philip P. Gray and the cooperation of Mrs. Frances Feerst who obtained most of the alpha-amylase data on the flours.

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BOOK REVIEWS

Experiments in Biochemistry. By Max S. Dunn and William Drell, 1951. First edition, v + 197 pp. McGraw-Hill Book Co., Inc. Price, $6.00.

Although there are available to students in beginning biochemistry a number of texts dealing with laboratory methodology, this book is unique in that it may serve not only as a laboratory guide but also as a comprehensive reference source for biochemical subject matter. The experiments have been selected to enable the student to become proficient in the synthesis, isolation, purification, and analysis of a wide variety of biological substances. Typical experiments include the synthesis of phenylalanine, synthesis of a peptide, asymmetric enzymatic synthesis of an optically active amino acid derivative, isolation of a sterol, isolation of cystine, determination of ascorbic acid, determination of alkaline phosphatase activity, and others. Schemes are also presented for the identification of amino acids and carbohydrates and the characterization of lipides. In the performance of these experiments ample opportunity is presented for the student to become familiar with the operation of special instruments such as the photoelectric colorimeter, pH meter, and polarimeter and with special techniques which include microbiological assays (nicotinic acid and choline), paper chromatography (purines and pyrimidines), and animal assay (vitamin D with chicks).

Each experiment is prefaced with a brief historical introduction and such other information which is pertinent to an understanding and appreciation of the experiment. The experimental procedure is detailed in a clear explicit manner. Where special techniques are involved reference is sometimes made to further details in the appendix. In addition each experiment is generously annotated for the purpose of giving the student a more detailed explanation of underlying principles, chemical reactions, and related current investigations. Original references are extensively cited, and therein lies one of the most useful features of this book.

Directions for the preparation of special reagents, complete lists of apparatus and reagents required, first aid measures, and a summary of the results obtained in the authors' classes in previous years are included in the appendix. The thoroughness of the literature coverage is evidenced by the more than 900 references which are given in the bibliography. A valuable adjunct to this book is the inclusion of sample data sheets to illustrate the manner in which the data should be recorded by the student.

Only a few typographical errors were in evidence. A parenthesis was omitted in line 2 of p. 99. The authors obviously had in mind the word "illumination" in the sentence (p. 100): "chicks will not eat properly if the *illustration* is inadequate." The authors have misquoted the original reference in the statement (p. 95): "the lethal dose of choline (for the rat) is 29–75 μg. of choline chloride per 100 g. of body weight." In the original paper cited, 29–75 *mg.* were the figures given.

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Soybeans and Soybean Products. Edited by Klare S. Markley. Volumes I and II. v + 1145 pp. Interscience Publishers, Inc., New York. 1950. Price, $11.00 per volume.

In the wake of the phenomenal growth of the soybean industry in this country, an enormous amount of literature has accumulated. This book attempts to satisfy the need for a comprehensive and unified treatment of all the known information pertaining to the many diversified aspects of soybean technology and utilization. To this end, the collaborative efforts of 26 experts have been combined to produce a two-volume book composed of 25 chapters each dealing with one of the numerous specialized fields encompassed by the soybean or its derived products.

The four main sections into which the book has been divided—production, structure and composition, processing, and utilization—illustrate the broad scope of the material which is covered. The production of soybeans is described in two chapters, one dealing with environmental factors affecting the growth and cultivation of the soybean plant, and the other with the economic aspects of the soybean in world trade. The section on structure and composition is comprised of chapters embracing such subjects as the botanical characteristics of the soybean plant, the chemical composition and physical properties of the seed and its oil, and the nutritive factors in soybean products. The gamut of operations involved in the processing of soybeans is described in chapters devoted to grading, handling, storage, and the mechanical and solvent extraction of the oil and its subsequent treatment. The section on the utilization of soybean products reveals a truly amazing number of uses for the soybean both as edible products for man and farm animals and as valuable ingredients in a variety of industrial processes.

From the point of view of consistency, the references pertaining to chapter 1 by Morse on the "History of Soybean Production" should have been included in the text in the form of footnotes instead of at the end of the chapter. This would have conformed to the procedure used in all the other chapters. On page 394, Mitchell in his chapter on the "Nutritive Factors in Soybean Products" (chapter 10) states that "the availability of the proteins of the raw soybean has not been extensively studied and in man not at all." Yet, four pages later, he refers to nitrogen-balance experiments with humans in which autoclaved soybean flour was compared with raw soybean flour. The title of Deuel's chapter, "Nutritional Value of Soybeans and Soybean Products" (chapter 18), is somewhat misleading inasmuch as it deals primarily with the nutritional aspects of fats and only incidentally with soybean oil. It would have been logical to expect this chapter to have followed the one by Mitchell, instead of 8 chapters later. Mitchell very obligingly omitted soybean oil as one of the nutritive factors in soybean products.

Although an overlapping of subject matter is unavoidable in a collaborative effort of this nature, the duplication of material has been reduced to a surprising minimum. This book should be met with eager acceptance by those who are concerned with any one of the many facets of the soybean industry.

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Advances in Agronomy. Vol. III. Edited by A. G. Norman. 361 pp. Academic Press Inc., New York, New York. 1951. Price, $7.50.

The third volume of the series contains eight chapters, three dealing with soils, three with crop management and two with reviews of crop and soil research.

The first chapter of 90 pages is a treatise on "The Subsoil" and was written by Eric Winters, University of Tennessee, and Roy W. Simonson, U.S.D.A. It deals with the nature and properties of subsoils of the Great Soil Groups and includes discussions of the effect of temperature on subsoils, relationships of subsoils to plant growth and the management of exposed subsoils. It is of primary interest to soil scientists.

The second chapter covers the subject of management of alfalfa meadows and was prepared by C. J. Willard of Ohio State University. The chapter consists of only sixteen pages but covers the subject in a remarkably thorough fashion. It is divided in six major sections covering such subjects as the effect of cutting systems on the quantity and quality of hay; the effect of different systems of cutting on root reserves, sustained yields and longevity of stands; the relation of various factors to time of cutting; cultivation of established alfalfa; weed control; and practical recommendations for cutting. The chapter will be of interest to both agronomists and growers.

The third chapter, "Soil Potassium," was written by R. F. Rutmeier, U.S.D.A., and is a technical discussion of the forms of soil potassium, the relationship which exists between it and other cations and the movement and distribution of potassium in the soil profile. The subject matter of the chapter has been oriented toward the fundamental principles of the chemistry of soil potassium. This chapter will be of especial value to the soil scientist.

The fourth chapter entitled "The Changing Pattern of Agriculture in England and Wales" was prepared by J. A. McMillan of the National Advisory Board, Ministry of Agriculture and Fisheries, London. It reviews the changes in the agricultural pattern in the two countries from 1921 to 1938. This period, as the author emphasizes, was one of stress, with a world war and some years of an uneasy peace. The chapter emphasizes the effect of economics and changing times on agricultural patterns and is prophetic of changes to come in the future.

The next chapter, written by Glenn W. Burton, U.S.D.A., Tifton, Ga., deals with the adaptability and breeding of suitable grasses for the southeastern states. It gives consideration to seventeen grasses grown in the area. The author reviews the history of the development of agriculture in the southeastern United States and discusses the problem of fitting adaptable grasses to specific environments. To do this the characteristics of the grass as well as the environment are important. Such matters as improvement by breeding, culture and fertilization are reviewed.

The sixth chapter is devoted to chemical treatment of soils for nematode control and was written by A. L. Taylor, U.S.D.A., Beltsville, Md. Since nematode control is becoming increasingly important the treatise is a timely one. It covers the matters of soil fumigation by different materials and the effect of fumigants on the soil. The chapter will be of especial interest to horticulturists, gardeners and greenhouse operators.

The seventh chapter was prepared by Horace J. Harper of Oklahoma Agricultural and Mechanical College and is devoted to problems and progress of soil conservation. The author emphasizes the importance of education and the part it plays in getting conservation on the land. He also discusses the objects of conservation research, dealing with the part played by specific erosion control practices and soil and crop management. Economic problems and problems of social adjustment are considered. The chapter should be of wide interest.

The eighth and final chapter is devoted to irrigation agriculture and soil research in the U. S. Prepared by R. O. Parks, U.S.D.A., Beltsville, Md., it deals with the historical development of irrigation in the United States and progress in the modern period. Developments in such projects as the Columbia River Basin, the Missouri Basin, and the Arkansas-White-Red River Basin are reviewed. An interesting feature is a section dealing with the future of irrigation in which it estimated that only a little more than half of the land suitable for irrigation is now being irrigated.

The volume as a whole is a highly creditable one, well organized and well documented.

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SUGGESTIONS TO AUTHORS

General. Authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* **6**: 1–22. 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Editorial Style. A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°–10° C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B+C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

For more detailed information on manuscript preparation see *Cereal Chem.* **29**: 89–90 (1952).

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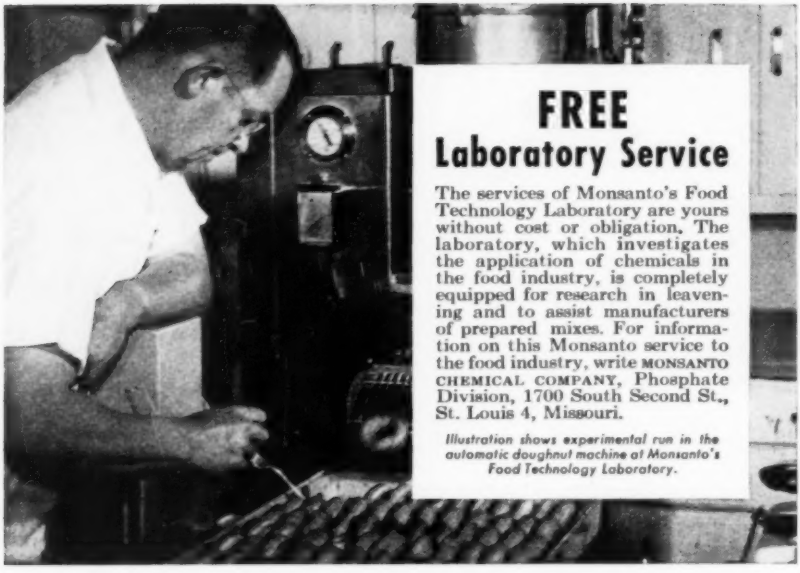

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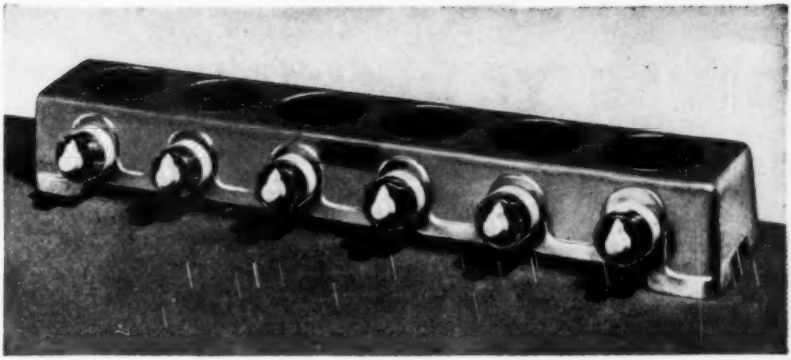

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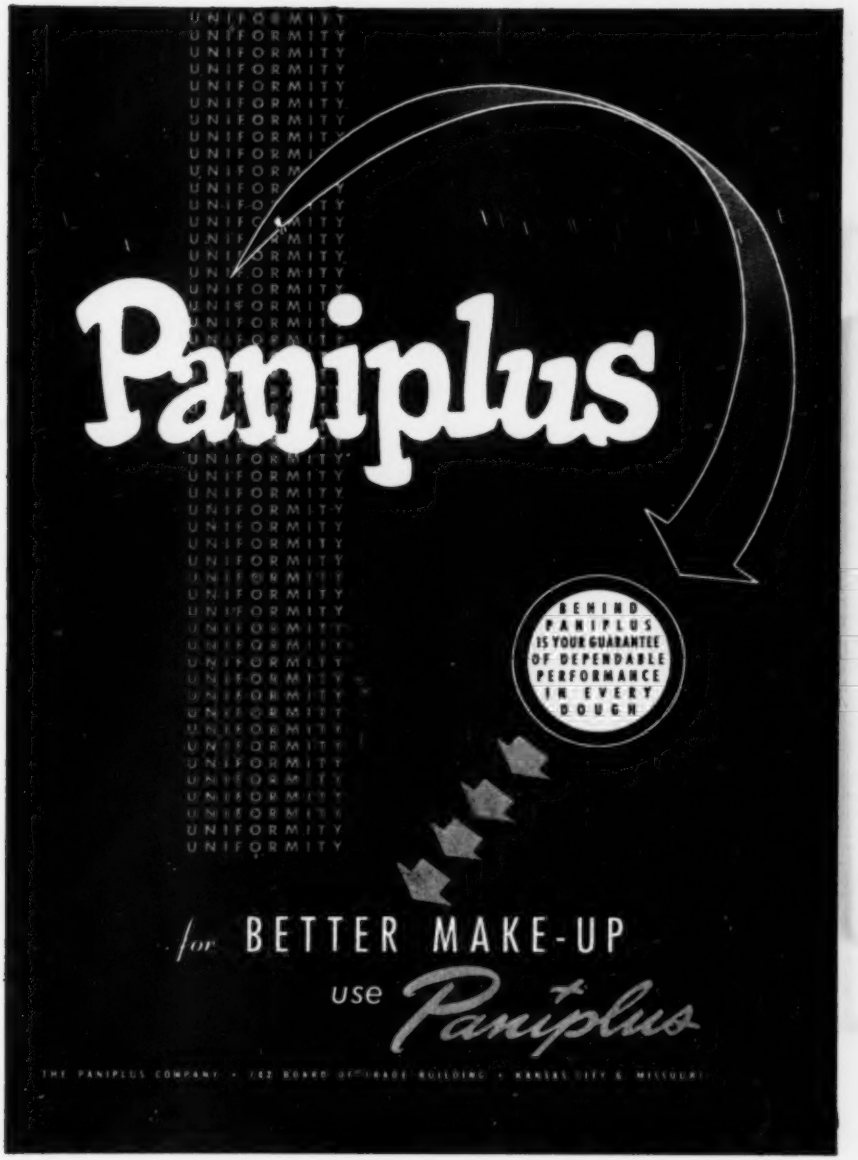
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